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(54) Title: METHODS AND COMPOSITIONS FOR MODULATING G PROTEIN ACTION

(57) Abstract

The present disclosure relates to methods and compositions for modulating the action of G proteins. G proteins are important to cellular regulation in all human cells and their function is involved in a wide variety of human disease processes. In particular aspects, the present disclosure concerns the development of mastoparan analogs as well as other amphipathic and cationic peptides, termed receptor-based analogues, that can promote activation of one or more G proteins, and are therefore proposed to be useful in the treatment of a variety of disorders including asthma, ulcers, cardiovascular diseases and even Parkinson's disease.

DESCRIPTION

METHODS AND COMPOSITIONS FOR MODULATING G PROTEIN ACTION

BACKGROUND OF THE INVENTION

1.   Field of the Invention

The present invention is directed towards methods and compositions of amphiphathic compounds used as drugs to directly regulate G protein function *in vivo*. More particularly, the present invention is directed towards amphipathic compounds based on the key structural determinants of modified mastoparan and receptor-derived peptides. Such compounds would be used as novel drugs to combat a variety of disease states in which G proteins are intimately involved, e.g., asthma, gastric ulcers, cardiovascular disease, allergies, Parkinson's disease, small cell carcinoma of the lung, and the like.

2.   Description of the Related Art

G proteins (guanine nucleotide binding regulatory proteins) are important to regulatory mechanisms operating in all human cells. Impairment of their function can perturb the cell's response to hormonal signals and adversely affect many intracellular metabolic pathways, thus contributing to the development and maintenance of a wide variety of disease states.

When functioning normally, G proteins act as an integral part of the signal transducing mechanism by which extracellular hormones and neurotransmitters convey

their signals through the plasma membrane of the cell and
thus elicit appropriate intracellular responses.

5   In its simplest terms, the signal transducing
mechanism can be said to comprise three distinct
components. A receptor protein with an extracellular
binding site specific for a given agonist, such as the β-
adrenergic receptor; a membrane-bound effector protein
that when activated catalyzes the formation or
10   facilitates the transport of an intracellular second
messenger, an example is adenylate cyclase which produces
cyclic AMP (cAMP); and a third protein which functions as
a communicator between these two. G proteins fulfill
this vital role as communicator in the generation of
15   intracellular responses to extracellular hormones and
agonists.

G proteins are composed of three polypeptide
subunits, namely Gα, Gβ and Gγ. The conformation of each
20   subunit and their degree of association changes during
the signal transducing mechanism. These changes are
associated with the hydrolysis of the nucleotide GTP to
form GDP and P_i (GTPase activity). The binding sites for
GTP, GDP and the GTPase catalytic site reside in the α
25   subunit.

The G protein cycle which occurs each time a signal
is conveyed across the membrane can be summarized as
follows:
30

In an unstimulated cell the G proteins are found in
the resting state in which α, β and γ are complexed
together and GDP is bound to Gα. The binding of an
appropriate hormone or agonist to the receptor changes
35   its conformation and causes it to activate the G protein
by displacing GDP and allowing GTP to bind. This is the
rate-limiting step of the G protein cycle. When GTP is

bound to Gα it may dissociate from βγ and is able to bind
to, and activate, adenylate cyclase which releases cAMP
into the cytoplasm. GTP is then hydrolysed to GDP and
the cycle is complete.

The series of complex interactions has evolved to
allow signal amplification, such that a single hormone-
receptor complex can trigger the production of several
hundred second messenger molecules, such as cAMP. cAMP
is a potent second messenger that binds to and activates
protein kinase A (PKA). PKA was first shown to play a
role in glycogen metabolism and is now known to influence
a variety of processes including transcription.

A further attribute inherent in this system is that
it allows several different receptors to interact with a
signal-generating enzyme. Some act in such a way to
activate the enzyme and some to inhibit it. This
involves distinct α subunits $G_{s\alpha}$ (stimulatory) and $G_{i\alpha}$
(inhibitory) that combine with the same βγ complex to
form stimulatory or inhibitory G proteins. An example of
a receptor that interacts with G_i to lower the
concentration of cAMP is the α_2-adrenergic receptor. The
integration of the signals from G_s and G_i is one of the
ways in which the level of cAMP in the cell can be fine-
tuned in response to several different extracellular
agonists.

Although G proteins were first identified and
characterized in relation to the adenylate cyclase
system, as discussed above, it is now apparent that they
are involved in many other aspects of cell signalling.
In particular, certain G proteins act in the signal
transducing pathways that activate phospholipase C. This
is a key enzyme that catalyzes the hydrolysis of
phosphatidylinositol 4,5-bisphosphate (PIP_2) to form
diacylglycerol (DG) and inositol 1,4,5-triphosphate (IP_3).

DG causes the activation of protein kinase C (PKC) which phosphorylates a certain sub-set of cellular proteins and modulates their activity. For example, PKC is important in controlling the intracellular pH and in the trans-
5   criptional activation of specific genes. IP_3 is a small water-soluble molecule that causes the release of Ca^{2+} from intracellular stores where it has been sequestered. CA^{2+} itself is a potent intracellular messenger that plays a vital role in several metabolic and homeostatic
10   pathways.

As has been shown, the importance of G proteins to the well-being of the cell cannot be stressed too much. It is not therefore surprising that any modulation of G
15   protein function can have catastrophic consequences. Such is the case in individuals who are genetically deficient in G_s, their decreased responses to many hormones cause impaired growth, mental retardation and severe metabolic abnormalities.
20

Cholera and pertussis toxins also exert their effects through G proteins. Cholera toxin catalyzes the irreversible modification of $G_{s\alpha}$, by ADP-ribosylation, which destroys its GTPase activity and locks it into an
25   active state. The resulting prolonged elevation in cAMP levels within the intestinal epithelial cells causes a large efflux of Na^+ and water into the gut, which can prove to be fatal. Pertussis toxin, made by the bacterium that produces whooping cough, alters the $G_{i\alpha}$
30   protein in a similar manner and prevents the inhibition of adenylate cyclase, thus also raising cAMP levels.

Following the identification of G proteins as important elements in many pathological conditions,
35   several attempts have been made to design effective treatment strategies. However, each particular method employed suffers from certain drawbacks.

Many drugs are currently directed towards the
hormone receptors themselves, such as the ß-adrenergic
agents used in the treatment of asthma. The usefulness
of this class of drugs is limited by the problems of
receptor desensitization and down regulation. In the
normal physiological state the amount of functional
receptor on a cell's surface is not constant, but is
modulated in response to the hormone level. Down
regulation of receptors is a general response to a high
level of circulating hormone or agonist. The reduction
of functional cell surface receptors desensitizes the
cell and higher concentrations of agonist do not elicit
an appropriately higher response. Any therapeutic agent
which involves binding to the receptor is therefore
partly flawed by the reduction in the number of receptors
which will subsequently occur. It is evident that a
downward spiral can result in which ever increasing doses
are required to obtain the same effect, and at each dose
the number of effective receptors would decline further.

The present invention seeks to by-pass the problem
of receptor down regulation by using novel compounds that
directly regulate G protein function.

Mastoparan (MP) is a peptide toxin from wasp venom
that has been shown to directly stimulate G protein
activation. MP is the prototype of a family of peptide
toxins, collectively known as mastoparans, that form
amphiphilic α helices. MP has been shown to stimulate
guanine nucleotide exchange by G proteins in a manner
similar to that of G protein-coupled receptors.

When MP is bound to a phospholipid bilayer, it forms
an α helix that lies parallel to the plane of the
membrane, with its hydrophobic face within the bilayer
and its four positive charges (3 lysyl residues and
terminal amino group) facing outward. G protein-coupled

receptors are also believed to display clusters of
positive charge near the inner surface of the membrane,
some of which are predicted to form amphiphilic helices.

These observations gave rise to the idea that
mastoparans could be used to directly regulate G protein
function *in vivo*, and so form the basis of a novel family
of drugs that would not suffer from the drawbacks of
receptor desensitization. Such G protein-targeted drugs
could also be used in cases where G protein mediated
responses are important but in which no manipulatable
receptor input is available.

This invention relates to the development of the
above idea and the intelligent modification of MP to
engineer features providing the optimum activity and
desired G protein specificity.

SUMMARY OF THE INVENTION

The present invention addresses these and other
shortcomings and disadvantages in the prior art by
providing novel peptides that may be employed to activate
one or more G proteins. It is proposed that the peptides
of the invention will find a variety of pharmaceutical
applications due to their action as G protein activators.
Numerous bodily actions, as noted above, are mediated by
G protein action. Thus, the inventors propose that the G
protein activator peptides of the present invention may
be employed to treat asthma, such as in aerosol
formulations, as nasal decongestants due to their α_1-
adrenergic agonistic action, and even potentially as H1
or H2 blockers.

In one aspect of the present invention, peptides
have been developed through modification of the

mastoparan sequence to identify those modifications that will either enhance the G protein-stimulatory activity of the mastoparan sequence, or improve its selectivity for one or more G proteins. It is a particular object of the invention to provide analogs that are capable of selectively stimulating the G_s protein in that this G protein is particularly involved in many β-adrenergic actions. However, it is clearly also an object of the present invention to provide peptide analogs that will have more selective actions against one or more of the other G proteins relative to mastoparan itself, such as G_t, G_z, G_q, G_h, G_i, and/or G_{11}. In any event, it is found that each of the G proteins tend to have a particular role in the modulation of cellular functions, and appear to be associated in many cases with peripheral nervous system action.

In particular embodiments relating to mastoparan analogues, therefore, the invention relates to peptides other than the naturally occurring mastoparans, MP, MP-A and MP-T (which have sequences of INLKALAALAKKIL, IKWKAILDAVKKVL, and INLKAIAAFAKKLL, respectively). In general, the mastoparan analogues of the present invention are defined as comprising from about 12 to about 26 amino acids in length, that have incorporated one or more of the various modifications proposed by the inventors and found to result in a useful analogue. In this aspect, the peptides will within their structure comprise a mastoparan analog nucleus region having the following general formula:

$$AA_1-AA_2-AA_3-AA_4-AA_5-AA_6-AA_7-AA_8-AA_9-AA_{10}-AA_{11}-AA_{12}$$
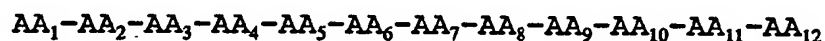
wherein:

AA_1 = L, W, A, F, Y or W;
AA_2 = K, A, R or Q;

AA_3 = A or K;

AA_4 = L, W, I, A, K or F;

AA_5 = A, L, K, C or R;

AA_6 = A, D or K;

5 AA_7 = L, C, W, A, F or K;

AA_8 = A, V, K or R;

AA_9 = K, R, Q or A;

AA_{10} = K, A, R or N;

AA_{11} = L, I, W, V, A or K; and

10 AA_{12} = L, A or C; or

Although naturally occurring MP, MP-A and MP-T each
have 14 amino acids in their structures, the inventors
have discovered that G protein modulating peptides may be
15 longer or shorter than this, so long as the MP analogue
peptides include the 12 amino acid nucleus discussed
above. It will be appreciated that the foregoing general
structure may be aligned with the MP, MP-A, and MP-T
structures by comparing AA_1 with the third MP amino acid
20 from the amino terminus, AA_2 with the fourth MP amino
acid, and so on. However, in more preferred embodiments,
the peptides of the present invention will comprise a 14
amino acid mastoparan analogue region within their
structure, wherein the mastoparan analog region has the
25 following structure:

$$AA_A-AA_B-AA_1-AA_2-AA_3-AA_4-AA_5-AA_6-AA_7-AA_8-AA_9-AA_{10}-AA_{11}-AA_{12}$$

wherein:

30

AA_A = I or C;

AA_B = N, K, Q or L;

AA_1 = L, W, A, F, Y or W;

AA_2 = K, A, R or Q;

35 AA_3 = A or K;

AA_4 = L, W, I, A, K or F;

AA_5 = A, L, K, C or R;

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AA_6 = A, D or K;

AA_7 = L, C. W, A, F or K;

AA_8 = A, V, K or R;

AA_9 = K, R, Q or A;

5   AA_{10} = K, A, R or N;

AA_{11} = L, I. W, V, A or K; and

AA_{12} = L, A or C.

A large number of peptides bearing a mastoparan

10   analogue region, and falling within the foregoing general
structure, have been synthesized, tested and shown by the
inventors to have G protein modulatory activity. These
peptides include INLKALAALAKKLL, INLKALAALAKKLA,
INLKALAALAKKAL, INLKALAALAKKKL, INLKALAALAKALL,

15   INLKALAALAKRLL, INLKALAALAAKLL, INLKALAALARKLL,
INLKALAALKKKLL, INLKALAAAAKKLL, INLKALAAKAKKLL,
INLKALAKLAKKLL, INLKALKALAKKLL, INLKAAAALAKKLL,
INLKAKAALAKKLL, INLKKLAALAKKLL, INLAALAALAKKLL,
INLRALAALAKKLL, INAKALAALAKKLL, INFKALAALAKKLL,

20   INLKAFAALAKKLL, INLKALAALAKALL, INLKALAALARALL,
INLKALAALAQALL, INLAALAALAKALL, INLKALAALAAALL,
INLRALAALAKALL, INLRALAALARALL, INLQALAALAKALL,
INLQALAALAQALL, INLKALKALAKALL, INLQALKALAKALL,
IQLKALAALAKALL, INYKALAALAKKIL, INYKALAACAKKIL,

25   INYKALCALAKKIL, CNYKALAALAKALL, INYKALAALAKALC,
INLKALAALAKNLL, ILLKALAALAKALL, INLRALRALARALL,
INLKALAALKKALL, INWRAWRAWARAWL, INLRALAALRRRLL,
INLKALAALKKKLL, CLLKALAALAKALL,
LTAVLLTLLLYRINLKALAALAKALL. Numerous of these are shown

30   to be particularly active and/or selective, as disclosed
in more detail below (see, e.g., Table IV hereinbelow).

In still further embodiments, the present invention
relates to peptides developed through a consideration of

35   various receptor sequences found by the inventors to
comprise useful G protein modulatory sequence regions.
The receptors studied for this purpose include the turkey

β-I3N, β-I2, β-I3C, β-I4N; the M1-I3N; Ha-α1-I3N; Ha-β2-I3N; M1-I2; M2-I3N; and the cow A1-I3N. From these studies, the following peptides were identified to comprise G protein modulatory regions, and can thus be employed in the construction of shorter or longer peptides as G protein modulators in accordance with the invention: CVYREAKEQIRKIDRVL; CVYREAKEQIRKIL; IVYREAKEQIRKIDRVL; IVYREAKEQIRKIL; CIYRETENRARELAALQGSET; CIYRETENRARELAALQGSETIL; CVYIVAKRTTKNLEAGVMKEIL; CVYIVAKRTTKNLEIL; Acetyl-CVFQVAKRQLQKIDKVL; CPLSYRAKRTPRRAALM; CPFRYQSLMTRARAKVI; REHKALKTLGIIC; CRSPDFRKAFKRLLC; CVYREAKEQIRKIDR; CISRASKSRIKKDKKEPVAIL; CISRASKSRIKKDKKIL; CVYVVAKRESRGLKSGLKTDIL; or CVYVVAKRESRGLKIL.

Still further embodiments of the present invention reflect the inventors' discovery that the presence of relatively hydrophobic amino acids at the amino terminus of the selected peptide will provide an enhancement of G protein stimulatory activity. Preferred hydrophobic terminal amino acids include isoleucine, leucine and valine. However, it is proposed that other hydrophobic amino acids such as phenylalanine, tryptophan or methionine can be employed as well.

The inventors have also determined that particular advantages in terms of pharmacologic efficacy will be realized where the peptide has been modified at one or more of its termini to "protect" the peptide against degradation and thereby enhance it action. A convenient means of protecting the peptide is through amidation or esterification of its carboxy terminus or acylation of its amino terminus. Carboxy terminal amidation of peptides has generally been found to be required for obtaining activity. Therefore, prior to formulation for administration to patients, one will desire to provide the peptide in amidated form. The effects of amino

terminal acetylation are more variable. It is more
preferrable to add larger alkyl groups to the amino
terminus, such as by attachment to a cysteine residue
using n-iodoalkanes. The inventors have found that
5   alkylation of such a cysteine residue employing, e.g.,
C12 or C16, results in a significant increase in potency
of both the mastoparan-based activating peptides, as well
as the receptor-based peptides, discussed above. While
preferred embodiments employ C12 or C16, the present
10   invention contemplates that the certain benefits may be
achieved through alkylation of the amino terminus with
any convenient hydrophobic alkyl group, such as C10-C22,
C12-C18, etc. The reason for this enhancement of potency
is not clear, but could be due to enhanced binding to the
15   membrane surface and resultant stabilization of an
amphipathic structure which may not be fixed when the
peptide is in aqueous solution.

     The inventors have also determined that an enhanced
20   hydrophobicity of the C-terminus tends to have beneficial
effects as well. An exemplary approach to enhancing the
hydrophobicity of the C-terminus is through the addition
of short hydrophobic peptide regions, such as a
hydrophobic dipeptide region. As mentioned above,
25   exemplary hydrophobic amino acids which may be added to
the C-terminus include isoleucine, leucine and valine.
However, the invention is certainly not limited to these
hydrophobic amino acids.

30   In still further embodiments, the present invention
concerns peptides that incorporate one or more D-isomers
of amino acids. D-isomers are generally less susceptible
to biological degradation, and therefore should exhibit
an increased half life in the body. The inventors have
35   found that peptides constructed using D-isomers of amino
acids nevertheless exhibit G protein modulatory activity,

and therefore can be employed in connection with the present invention.

The inventors have further determined that various general structural changes in the basic mastoparan structure can be employed to enhance G protein stimulatory activity. For example, the replacement of the lysine at position 12 yields a dramatically increased activity as an activator of G_i and G_o. In fact, increases in activity can be greater than 10-fold by the replacement of lysine at position 12 with alanine. From this general observation, the inventors have found that the replacement of AA_2, AA_9 or AA_{10} (corresponding to positions 4, 11 and 12 of mastoparan, respectively) with amino acids such as alanine, glutamine or arginine can result in peptides having an improved pharmacology. Furthermore, the inventors have found that where at least two of these residues comprise an alanine, glutamine or arginine, then particular advantages are achieved, generally in terms of enhanced potency and maximal effect.

Of course, in that the peptides of the present invention are intended for administration to mammals, particularly humans, the present invention concerns embodiments in the preparation of pharmacologic compositions which comprise a therapeutically effective amount of one of the foregoing described G protein modulatory peptides, dispersed in a pharmacologically acceptable carrier or diluent. Since the peptides of the present invention will find their greatest utility through topical administration, such as in the treatment of asthma, ulcers, glaucoma, respiratory tract congestion or inflammation, and the like, pharmaceutical compositions of the present invention will preferably employ a carrier that is adapted for topical administration. For ophthalmic applications, one would

desire to employ a sterile solution. In any event,
pharmaceutical compositions of the present invention will
generally include acceptable salts and/or buffers for
maintaining a selected pH, such as between about 6 and 8,

5   and more more preferably between about 7 and 7.5. For
administration as inhalants, such as in the treatment of
asthma, one may also desire to formulate the composition
in a manner to allow its administration in the form of an
aerosol.

10

As a further matter, it is pointed out that the
inventors propose that the peptides of the present
invention will find additional application as structural
models for computer-based analysis leading to the design

15   of non-peptidyl agents that will mimic the effect of
these peptides.

BRIEF DESCRIPTION OF THE DRAWINGS

20

Fig. 1. Stimulation of GDP/GTP exchange by analogs
of MP. The release of $[\alpha-^{32}P]$GDP from G_i was measured at
30°C in the presence of the concentrations of several
different MP analogs whose structures are listed in Table

25   I: O, Mas7; △, Mas8; ▲ Mas9; □, Mas19; ●, MP; ■, MP-X;
∇, MAS14; ▼, Mas6. First order dissociation rate
constants (k_{diss}) were determined from duplicate four-point
time courses as described under "Experimental
Procedures."

30

Fig. 2. Circular dichroism spectra of active and
inactive MP analogs. Spectra were determined as
described under "Experimental Procedures" in the presence
or absence of PC. Molar ellipticities are calculated per

35   mol of amino acid residue. Mas7 alone, ——; Mas7 plus
PC, — — —; Mas 17 alone, · — · —; Mas17 plus PC, ----.

     Fig. 3. Inhibition by BAC of the MP-stimulated
GTPase activity of G_i. The GTPase activity of
reconstituted G_i (19.6 fmol/assay) was measured at 30°C
with 200 nM GTP in the presence of 0.1 mM free Mg^{2+} ($\square$).

5   AT 15 min, Mas7 (10 μM final) was added to one of the
assay mixtures ($\blacksquare$) and, at 35 min, BAC (3 μg/ml) was also
added to a portion of this assay mixture ($\triangle$). Another
assay was initiated in the presence of 3 μg/ml BAC (O,
and 10 μM Mas7 was added to a portion of this assay

10   mixture at 20 min ($\bullet$).

     Fig. 4. Inhibition by BAC of MP-stimulated GDP
exchange by G_i. A, the dissociation of [α-^{32}P]GDP from 4
nM G_i was measured at 30°C as described In Example 1 in

15   medium that contained 0.1 mM free Mg^{2+}, the concentration
of BAC shown on the *abscissa*, and the following
concentrations of Mas7: $\bullet$, O; $\triangle$, 1 μM; $\blacktriangle$, 3 μM; $\square$, 10 μM;
0, 20 μM; $\blacksquare$, 30 μM; , 100 μM. Samples were taken
between 5 and 120 s, as appropriate, and first order

20   exchange rate constants, k_{obs} were obtained by fitting four
pairs of duplicate data points. B, the data from *panel A*
were replotted to show the response to increasing
concentrations of Mas7 at the increasing concentrations
of BAC. Hill coefficients for stimulation were 2.9 ±

25   60.8, 3.1 ± 0.7, 3.3 ± 0.7, 2.6 ± 0.9, 2.1 ± 0.4, and 2.2
± 0.4 at 0 (O), 0.1 ($\bullet$) 0.3 ($\triangle$), 1 ($\blacktriangle$), 3 ($\square$), and 10 ($\blacksquare$)
μg/ml BAC. The inset shows the change in the
concentration of MP7 that produced half-maximal
stimulation of GDP exchange (EC_{50}) at each concentration

30   of BAC.

     Fig. 5. Stimulation of the steady state GTPase and
GDP exchange activity of G_o by BAC. The GTPase activity
of 0.93 nM G_o in PE/PC/PS vesicles (3:4:3) was assayed at

35   100 mM [γ-^{32}P]GTP in the presence of 0, 1, 3, 10 and 30
μg/ml BAC. Activities are shown as molar turnover
numbers (O). Rates were taken from linear portions of

-15-

the time course ranging from 1 to 20 min. For 100, 300, and 1000 μg/ml BAC (Δ; shown at the right), activities were not linear at 1 min. The release of [α-^{32}P]GDP was also measured under similar conditions. These data are
5   expressed as first order rate constants ($\bullet$).

     Fig. 6.   Effect of MP on the binding of acetylcholine to reconstituted muscarinic receptor. Purified muscarinic cholinergic receptor and G_o were co-
10   reconstituted into phospholipid vesicles, and the binding of acetylcholine (Ach) was measured by competition with the antagonist [^{3}H]QNB as described in Example 1. Assays contained 3.8 nM [^{3}H]QNB. Assays contained, in panel A: 30 μM MP ($\bullet$) or no addition (O); in panel B: 30 μM Mas 17
15   ($\bullet$), 10 μM GTPγS (Δ), or no addition (O); and in panel C: 30 μM Mas7 ($\bullet$), 10 μM GTPγS(Δ), or no addition (O). Panel D shows the binding of acetylcholine to receptors reconstituted into vesicles without G protein $\bullet$, 30 μM MP; Δ, 10 μM GTPγS; O, no addition. The data represent
20   the means of duplicate determinations of specific binding.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS
25

Introduction

     G proteins are important to regulation in all human cells and their function is involved in a wide variety of
30   disease processes. Signaling through G proteins is usually initiated by receptors for hormones, neurotransmitters and other signaling molecules. Higashijima and co-workers (Higashijima et al., 1987) first suggested that mastoparan, a peptide toxin from
35   wasp venom, directly stimulates G protein activation. This suggestion was confirmed in 1988 (Higashijima et al., 1988) and further data indicated that mastoparans

stimulate G protein activation through a mechanism very
similar to that used by receptors (Higashijima et al.,
1990). Mastoparan is the prototype of a family of about
10 peptide toxins isolated from related wasps (known

5   collectively as mastoparans). The present inventors have
since synthesized and tested numerous peptides that are
amphipathic and cationic and can promote G protein
activation. These peptides have been synthesized based
on a consideration of mastoparan structure, as well as

10   the structure of various G-protein-linked receptors such
as several isoforms of $\alpha 1$, $\alpha 2$, β-adrenergic, muscarinic 1
and 2 and dopamine D1 receptors. The peptides of the
present invention, which are based on mastoparan and
receptor sequences, are generally considered to be

15   activator peptides in that they activate the action of
one or more G proteins.

At the core of the present invention, therefore, is
the finding that amphipathic compounds can be used as

20 .   drugs to directly regulate G protein function, thereby
manipulating disease states in which G proteins are
significant. G proteins are diverse and are intimately
involved in numerous diseases (asthma, ulcers, many
cardiovascular diseases, Parkinson's disease) and are at

25   least peripherally involved in most physiological
regulatory processes. Furthermore, these amphipathic
compounds can either activate G proteins or block their
activation by receptors.

30   The present invention relates in part to the ability
of mastoparans and receptor peptides to regulate G
proteins directly. This can allow the development of
drugs to treat diseases either by promoting the
activation of specific G proteins or by blocking their

35   activation by receptors. Because the mastoparans and
congeners can fulfill these functions, the inventors
propose that these agents will prove useful as a new

class of therapeutically useful drugs. Activator
peptides of the present invention are highly efficacious:
peptides of the present invention can frequently achieve
in vitro stimulation of G protein activation far greater
than that caused by receptors. The use of these peptides
would largely obviate receptor desensitization and down-
regulation, which frequently limit the utility of
receptor-directed drugs. G protein-targeted drugs could
also be used in cases where G protein mediated responses
are important but in which no manipulatable receptor
input is available.

Furthermore, the use of G protein-targeted drugs
forgoes the exquisite selectivity of receptor-directed
drugs. This might initially limit the use of the
compounds to topical or local applications because of
potentially untoward effects of stimulating G protein
functions in non-target tissues. Moreover, the
susceptibility of peptides to hydrolysis by cellular and
serum peptidases and proteases could serve to compromise
the duration of action of the present activator peptides.
However, in that the inventors have found that stable
all-D-isomers of mastoparan are also active, such agents
can likely be employed to circumvent problems of
hydrolysis and susceptibility to enzymatic degradation in
that most enzymes are specific of L-isomeric substrates.

Specific Applications

To be an effective drug, an activator peptide must
be adequately specific for the G protein to be regulated.
The mastoparans themselves show some specificity. For
example, mastoparan is most effective as an activator of
G_o, G_z and G_i. In the inventors' initial screen of
systematically modified mastoparan congeners it was found
that many changes in the mastoparan structure slightly
changed the selectivity of mastoparan between G_i and G_o by

less than 5-fold. Such modifications included, e.g.,
those represented by analogs Mas4, Mas5, Mas11, Mas16,
and Mas17 (see Table IV). However, several of these
compounds also had significant effects on G_s, including
ER21 and ER30 (see Tables V and VI).

A variety of applications are proposed for the G
protein activator peptides of the present invention. For
example, due to their β-adrenergic activity, a G_s-directed
activator peptide could be used as an aerosol
bronchodilator to treat asthma. A number of peptides set
forth below (see Tables IV and V) have significant G_s-
specific action. Examples include C12-ER18, C12-ER19,
ER36 and even ER40 (Table V). Asthma is a widespread
disease that is not treated adequately by β-adrenergic
drugs (that ultimately work via G_s). Topical application
seems particularly appropriate for asthma treatment, and
is currently used for other anti-asthma drugs. Potency
should not generally be a problem because of the route of
application. Intense pharmacologic intervention during
the initial spasmic phase of asthma is particularly
valuable because limitation of initial spasm seems to
delay or diminish the delayed inflammatory asthmatic
response.

The peptides of the invention should also find
application as nasal decongestants, which are generally
α_1-adrenergic agonists. The α_1-adrenergic agonists
promote activation of G_q and/or G_{11} (or, perhaps, a newly
identified G protein known as G_h). Direct activation of
these proteins might have the advantages discussed above
for bronchodilators. Furthermore, β-adrenergic
antagonists and muscarinic agonists are widely used in
the treatment of glaucoma, although their effectiveness
is limited. It is proposed that the peptides of the
present invention will be applicable to the treatment of
glaucoma, such as when formulated into an ophthalmic

dosage form for topical application. For example, a
peptide that activates G_q would be a far more active agent
than the muscarinic agonists, to which tolerance develops
rapidly. A peptide that blocks the activation of G_i might
5   do far better than the currently used β-adrenergic
antagonists.

Both H1 and H2 anti-histamines are currently of
great therapeutic importance: H1 blockers to treat nasal
10   allergic reactions and H2 blockers to treat gastric
ulcers. These receptors work on G proteins that have not
been unambiguously identified. However, these diseases
present readily accessible sites for topical application
and do not require dissemination throughout the body.
15

Surprisingly, small cell carcinoma of the lung is
best treated (although not well) by methadone, a
selective opiate agonist. Although this is clearly a
somewhat speculative application, stimulation of the
20   signaling pathway in these cells might add to the
pharmacologic treatment of these tumors.

Structural Determinants of the Function of Activating
25   **Peptides**

Mastoparan-Based Peptides

The inventors have synthesized and tested a large
30   number of peptides for their ability to activate G
proteins or to block activation. From these studies, a
number of trends in terms of the structure-activity
relationship (SAR) of such peptides have been observed.
For example, in referring to the natural mastoparan
35   sequence, the inventors have found that lysine 4 and
lysine 11 may be replaced by glutamine, arginine, or
alanine with little if any negative effect and, in some

cases, slight positive effect. However, replacement of
lysine 12 by alanine yields dramatically increased
activity as an activator of G_i and G_o. A number of
studies have been carried out by the inventors to confirm
5 the generality of this observation. In fact, increases
in activity can be $\geq$10-fold when tested at suitable
concentrations and in suitable media. For this reason,
mastoparan congeners having an alanine at the position 12
are particularly preferred.

10
The inventors have also found that the addition of a
lysine residue at position 10 has little effect on the
activity of mastoparan but greatly diminishes its lytic
capacity, in some cases by more than 100-fold. This is
15 extremely important in decreasing toxic, irritant and
inflammatory properties of mastoparan-based peptides.
Thus, lysine 10, alanine 12 mastoparan is the most
effective and least toxic G_o activator available.

20
A variety of structure modifications have been
studied by the inventors to determine which, if any, have
a significant pharmacologic effect. It has somewhat
surprisingly been found that carboxy-terminal amidation
of mastoparans is required for activity. The reason for
25 this is unclear but could be due to the need for a
hydrophobic carboxy terminus. However, the effects of
amino terminal acetylation are somewhat more variable.
For peptides that activate G_i and G_o, acetylation
decreases activity by 50-70%. Little effect of
30 acetylation has been observed for the peptides that
activate G_s.

While the pharmacologic effect of amino terminal
acetylation tends to be somewhat variable from peptide to
35 peptide, the inventors have found that alkylation of the
amino terminus with higher alkyls, such as C12 to C16,
can have a dramatic effect. Numerous means of amino

terminal alkylation are known in the art, and it is
proposed that virtually any such means as are known in
the art may be employed in connection with the invention
and obtain the advantages of the invention. The

5   inventors have rountinely employed the addition of a
cysteine residue, in that amino acids such as cysteine
may be alkylated readily. Moreover, the addition of a
cysteine residue at position 1 has little effect on
activity in the presence of a sulfhydryl compounds.

10   Thus, the addition of such a cysteine residue is
preferred where amino terminal alkylation is contemplated
in that the cysteine provides a ready substrate for
alkylation. Alkylation (C12, C16) of this cysteine
residue using n-iodoalkanes increased the potency of

15   mastoparan-based activating peptides as well as the
receptor-based peptides as well.

Studies of mastoparan analogues in which more than
one lysine residue is converted to alanine, glutamine or

20   arginine indicates that two positive charges (amino
terminus and one lysine) seem to be mandatory for high
activity and that three is generally better. Replacement
of lysine 4 with lysine at the 7 position (which should
also be on the hydrophilic face of the helix) is

25   generally without significant effect.

It has been shown by others that several novel
synthetic substance P antagonists can block the ability
of mastoparans to promote the activation of G_o and G_i

30   mediated events. They can also block stimulation in a
reconstituted system initiated by the M2 muscarinic
cholinergic receptor. Partial agonist activity of these
peptides is observed if they contain positive charge, but
a pentapeptide that contain D-tryptophan residues is a

35   full antagonist. This antagonist is effective on G_o and
G_i. It is also effective on G_s, although this interaction
is not yet well characterized.

Receptor-Based Peptides

The inventors have made a major effort to develop peptides that activate G_s, the G protein that stimulates adenyl cyclase. Among the mastoparan analogues synthesized so far, only leucine-2, alanine-12 activates G_s well ($\geq$ 10-fold). Others stimulate by less than 4-fold. In an attempt to identify more highly G_s active peptides, the inventors have synthesized several amphipathic peptides having a sequence designed based on a consideration of the third intracellular loop of various receptors. It has been postulated that the third intracellular loop is important for specifying a receptor's specificity and selectivity among G proteins (Wong et al., 1990, and the inventors' unpublished observations). Thus, a number of peptides of peptides have been synthesized and tested, and found to have significant action.

The unmodified receptor-based peptides, with or without carboxy terminal hydrophobic dipeptide extensions, were functionally inactive or only weakly active. When a cysteine residue was added at the amino terminus, and the sulfhydryl group alkylated, these peptides became potent and effective activators of G_s. They are among the most highly selective peptide activators that we have yet synthesized because they are only weakly active against G_o, G_i, G_z, and G_q. Several activate G_s more than 20-fold. These are the peptides what are proposed for use as either anti-asthma drugs or as the structural precursors for developing non-peptide anti-asthma drugs.

Required structural determinants for the receptor-based peptides so far are known to include a C-terminal hydrophobic dipeptide extension and amino terminal alkylation (C12 appears optimal). The inventors have not

systematically modified the internal sequences of the
receptor-based peptides as they have for the mastoparan-
based peptides, to further optimize activity,
selectivity, or potency. The preferred receptor-based
5   peptide is based on the sequence of the turkey
erythrocyte β-adrenergic receptor (e.g., peptides C12-
ER18, C12-ER19, C12-ER22, C12-ER23. C12-ER36).

        The dependence of a peptide's regulator activity on
10   its ability to form α-helices when binding to micelles or
bilayers should be stressed. This ability can be
determined from circular dichroic spectra of the
candidate peptide in the presence and absence of lipid
(Higashijima et al., 1990). This is of general
15   diagnostic importance, as indicated by more recent
studies of the G_s-directed β-adrenergic receptor-based
peptides carried out by the inventors. The peptides that
have not been acylated, and are thus inactive display CD
spectra characteristic of random coil both in aqueous
20   solution and in the presence of phospholipid vesicles.
Active (acylated) peptides give distinct α-helical CD
spectra only in the presence of bilayers, but not in
aqueous solution. The inventors propose that distinct
changes in CD spectrum caused by the addition of
25   phospholipid is a good first test for the ability of a
peptide to form an amphipathic structure when bound to a
membrane and to present its charged face to the aqueous
environment.

30

Peptide Preparation

        The conditions for preparation, alkylation and
purification of peptides of the present invention is well
35   within the skill of the art when considered in light of
the present invention. For peptide synthesis, the
inventors employ standard peptide synthesis technology

(e.g., using a solid phase peptide synthesizer), and the peptides may be readily purified by reverse-phase HPLC. The inventors routinely react the cysteine containing peptide with about two mole equivalents of the correct
5   iodoalkane in a slightly basic aqueous solution with a small amount of DTT. The addition of DTT has been found to increase the yield from about 30% to about 90%. In a typical protocol, the inventors employ approximately 1 μmol Cys-containing peptide, 2 μmol iodododecane (or
10   other iodoalkyl), 10 μmol DTT, 30 μmol base (KHCO$_3$), in 550 μl DMF and 150 μl H$_2$O. The reaction can be completed in 1 minute at 110°.

15   **Therapy and Pharmaceutical Compositions**

         For the treatment of the various disorders where one desires to modulate G protein function, it will generally be desireable to employ a dosage form and amount that
20   will be adequate to effect modulation of the selected G protein, without effecting appreciable effect on G proteins that are not associated with the targeted disorder or in non-target tissues that may contain the G protein target. Generally speaking, where a topical or
25   parenteral application is envisioned, one will desire to administer a dosage that will deliver an amount that will achieve an intracellular concentration of approximately 1 to 10 μM, as outlined in Example 2. Studies by the inventors using intact cells indicate that similar
30   concentrations in extracellular medium are efficacious. For a typical application as injectibles, aerosols, drops, creams, etc., the actual concentration will generally be adjusted to achieve this local concentration.
35
         The novel peptides of the invention are quite stable and may be administered alone or in combination with

pharmaceutically acceptable carriers, in either single or
multiple doses. Suitable pharmaceutical carriers include
diluents, sterile aqueous solutions, and the like.
Peptides of the present invention may not only be
5   advantageously employed for the preparation of
parenteral, topical and/or aerosol pharmaceutical
compositions for administration as described above, but
more particularly for the preparation of pharmaceutical
compositions suitable for use as ophthalmic solutions.
10   Such ophthalmic solutions are of principal interest for
the treatment of ophthalmic disorders such as glaucoma by
topical administration and the treatment of such
conditions in this manner is a preferred embodiment of
the present invention. Thus, for the treatment of
15   glaucoma the compounds of this invention are administered
to the eye of the subject in need of treatment in the
form of an ophthalmic preparation prepared in accordance
with conventional pharmaceutical practice, see for
example "Remington's Pharmaceutical Sciences" 15 Edition,
20 .   pages 1488 to 1501 (*Mack Publishing Co.*, Easton, Pa.).

Typical ophthalmic preparations will contain a
selected peptide or a pharmaceutically acceptable salt
thereof in a concentration that will conveniently achieve
25   a local concentration of about 1 to about 10 μM. Thus,
it is proposed that preparations of from about 0.001 to
about 0.1% by weight, preferably from about 0.0015% in a
pharmaceutically acceptable solution, suspension or
ointment, will find utility. Some variation in
30   concentration will necessarily occur, depending on the
particular compound employed, the condition of the
subject to be treated and the like, and the person
responsible for treatment will determine the most
suitable concentration for the individual subject.
35

Ophthalmic preparations will preferably be in the
form of a sterile aqueous solution containing, if

desired, additional ingredient, for example
preservatives, buffers, tonicity agents, antioxidants and
stabilizers, nonionic wetting or clarifying agents,
viscosity-increasing agents and the like. Suitable
5   preservatives include chlorobutanol, thimerosal and the
like.

       Therapeutic compositions of the present invention
will generally include suitable buffers include boric
10   acid, sodium and potassium bicarbonate, sodium and
potassium borate, sodium and potassium carbonate, sodium
acetate, sodium biphosphate and the like, in amounts
sufficient to maintain the pH at between about 6 and 8,
preferably between about 7 and 7.5. Suitable tonicity
15   agents are dextran 40, dextran 70, dextrose, glycerin,
potassium chloride, propylene glycol, sodium chloride,
and the like, such that the sodium chloride equivalent of
the solution is in the range 0.9 plus or minus 0.2%.
Suitable antioxidants and stabilizers include sodium
20 . bisulfite, sodium metabisulfite, sodium thiosulfite,
thiourea and the like. Suitable wetting and clarifying
agents include polysorbate 80, polysorbate 20, poloxamer
282 and tyloxapol.

25   Where desired, compositions may include suitable
viscosity-increasing agents include dextran 40, dextran
70, gelatin, glycerin, hydroxyethylcellulose,
hydroxmethylpropylcellulose, lanolin, methylcellulose,
petrolatum, polyethylene glycol, polyvinyl alcohol,
30   polyvinylpyrrolidone, carboxymethylcellulose and the
like. Ophthalmic preparations will be administered
topically to the eye of the subject in need of treatment
by conventional methods, for example in the form of drops
or by bathing the eye in the ophthalmic solution.
35

       The following examples illustrate preferred
embodiments of the present invention in terms of

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laboratory practices found by the present inventors to
work well in the practice of the invention. However, in
light of this disclosure, those of skill in the art will
appreciate that numerous alternatives may be employed
without departing from the spirit and scope of the
invention. Therefore, the present invention is not
intended to be limited to the specific methodology set
forth hereinbelow.

EXAMPLE 1

Regulation of G_i and G_o by Mastoparan, Related
Amphiphilic Peptides, and Hydrophobic Amines

MECHANISM AND STRUCTURAL DETERMINANTS OF ACTIVITY

This example relates to the interaction with, and
regulation of, G proteins by amphiphilic peptides. The
development of reproducible assay conditions for *in vitro*
measurement of peptide-stimulated nucleotide exchange has
enabled this interaction to be analyzed in detail. Using
this methodology it has been determined that mastoparan
(MP) stimulates guanine nucleotide exchange by G proteins
in a manner similar to that of G protein-coupled
receptors. This stimulation involves 1) MP stimulated
exchange by isolated G protein α subunits and $\alpha\beta\gamma$
trimers. Relative stimulation was greater with $\alpha\beta\gamma$
trimers and $\beta\gamma$ subunits could increase net MP-stimulated
activity. 2) MP action was enhanced by reconstitution of
trimeric G protein into phospholipid vesicles, with the
membrane-bound α-helical conformation of MP appearing to
be the activating species. 3) MP blocked the ability to
G_o to increase the affinity of muscarinic receptors for
agonist ligands, suggesting that MP and the receptor may
compete for a common binding site on G_o. 4) MP stimulated
steady state GTPase activity at < 1 μM Mg^{2+} and stimulated
the dissociation of both GDP and guanosine 5'-0-(3-

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thiotriphosphate) at < 1 nM Mg^{2+}. Millimolar Mg^{2+} blocked
the stimulatory effect of MP. This is in keeping with
the knowledge that both high and low affinity Mg^{2+} binding
sites are on the α subunit.

The details of the mechanistic studies are not
believed to be of particular importance to an overall
understanding of the present invention, but are
nonetheless incorporated herein by reference (Higashijima
et al., J. Biol. Chem., 265(24):14176-14186, 1990).

This example in particular relates to the structure-
activity relationships for the regulation of G_i and G_o by
MP, several related and unrelated biological peptides,
and a series of synthetic peptides designed to test the
importance of individual structural aspects of the MP
molecule.

EXPERIMENTAL PROCEDURES

Materials

MP was synthesized and purified as described by
Saito and his colleagues (*Chem. Pharm. Bull., 32*:2187-
2193, 1984, incorporated herein by reference). The MP
analogs listed in Table I were synthesized by standard
solid phase methods and purified by C-4 HPLC using an
acetonitrile gradient in 0.1% trifluoroacetic acid.
Analytical C-4 HPLC monitored at 220 nm showed less than
2% impurity in all samples. The identity of the peptides
was monitored by 400-MHz or 250-Mhz ^{1}H NMR spectroscopy
for MP and MP-X and by mass spectroscopy for the other
analogs. *Saacharomyces cerevisiae* α-mating factor,
calcitonin, and [D-Ala^2]Leu-enkephalin were prepared at
the Peptide Institute (Osaka) and gramicidin S, glucagon,
and compound 48/80 were purchased from Sigma. BAC was
purchased from Sigma, and other alkylammonium compounds

were from Aldrich. The critical micelle concentration of
BAC was measured in the buffer used for GTPase assays
(see below) using 1,6-diphenyl-1,3,5-hexatriene according
to the method of Schrock and Gennis, (*J. Biol. Chem,*
252:5990-5995, 1977, incorporated herein by reference).
The critical micelle concentration was approximately 20
μM.

[γ-^{32}P]GTP, [α-^{32}P]GTP, and [^{35}S]GTPγS were purchased
from DuPont-New England Nuclear. All lipids were
purchased from Avanti Polar Lipids, and other reagents
are from standard sources.

Protein Purification

G$_o$ was purified from bovine brain in the form of
resolved G$_o\alpha$ and $\beta\gamma$ subunits as described by Higashijima,
et al., 1987, (incorporated herein by reference), which
were mixed before each experiment at a 0.5 molar ratio
(α:$\beta\gamma$). Briefly, proteins were extracted from bovine
cerebral cortical membranes and chromatographed on DEAE-
Sephacel (Pharmacia LKB Biotechnology Inc.) according to
Sternweis and Robishaw, 1984, (incorporated herein by
reference), except that the column buffer contained AMF
to allow separation of α_o and $\beta\gamma$. Each pool was
chromatographed on Ultrogel AcA34 (LKB) in the presence
of AMF, diluted 4-fold in 20 mM Tris-Cl, pH 8.0, 1 mM
EDTA, 1 mM dithiothreitol, AMF, and applied to a 100-ml
column of heptylamine-Sepharose that was equilibrated in
the same buffer that also contained 25 mM NaCl and 0.25%
cholate. After the column was washed in the same buffer
that contained cholate and 100 mM NaCl, G proteins were
eluted with a 600-ml gradient from 100 mM NaCl plus 0.25%
cholate to 50 mM NaCl plus 1.3% cholate. Heptylamine-
Sepharose removed α subunits from the $\beta\gamma$ subunits and
helped remove α; from α_o.

Purification of α_o and $\beta\gamma$ was completed by chromatography on DEAE-Sephacel that was eluted with a gradient of 0-250 mM NaCl in the presence of 0.6% Lubrol and AMF. AMF was removed by chromatography on

5 hydroxyapatite and Sephadex G-25 as described by Sternweis, *et al.*, 1981, incorporated herein by reference. G_i was purified as the $\alpha\beta\gamma$ trimer from rabbit liver as reported by Bokoch, *et al.*, 1984, incorporated herein by reference. The G_i used in this study could be

10 recognized on a Western blot by anti-G_i-1 and anti-G_i-3 antibodies but not by anti-G_i-2 antibody, as documented by Mumby, *et al.*, 1983, incorporated herein by reference. Purified recombinant αi,1 was synthesized in *E. coli* prepared to the specifications of Linder, incorporated

15 herein by reference. During purification, G protein α subunits were assayed by [^{35}S] GTP$_\gamma$S binding according to the method of Higashijima, 1987, incorporated herein by reference). $\beta\gamma$ subunits were assayed according to their ability to deactivate Gs, as detailed by *Northup*, 1983,

20 incorporated herein by reference.

G proteins were reconstituted into phospholipid vesicles composed of dioleoyl-PC/bovine brain PS/1-palmitoyl,2-oleoyl-PE (3:4:3) as reported previously by

25 Higashijima *et al.*, 1988, incorporated herein by reference.

Muscarinic cholinergic receptor was purified from porcine brain as described by Haga and Haga, 1985,

30 incorporated herein by reference. To co-reconstitute receptors and G_o into phospholipid vesicles, receptor (5 pmol), G_o α subunit (100 pmol), and $\beta\gamma$ subunits (250 pmol) were mixed with 25 μg of a mixture of PE and PS (3:2, w:w) in 50 μl of 20 mM sodium Hepes, pH 8.0, 1 mM EDTA, 2

35 mM $MgSO_4$, 100 mM NaCl, 0.375 mg/ml deoxycholate, 0.075 mg/ml cholate and held at 0°C for 30 min. Each protein was added in less than 0.2 volume of the detergent-

containing solution in which it was purified, and the
lipid was added as a sonicated dispersion in the
reconstitution buffer. The mixture was chromatographed
on AcA34 as described previously (Higashijima, *et al.*,
5   1988) and vesicles were collected in the void volume.
Recovery of activity in the vesicles was typically 40%
for G_o and 8% for receptor, as compared to the work of
Haga, 1985; 1986, incorporated herein by reference.

10   *Assays*

Methods for the assay of steady state GTP hydrolysis
and GTPγS binding have been described previously by
Higashijima, 1987, and Ferguson, 1986, and incorporated
15   herein by reference. Unless noted otherwise, assays of
steady state GTP hydrolysis and of the binding and
dissociation of guanine nucleotides were carried out in
the buffer described previously, but without detergent
and with 1.1 mM $MgSO_4$ and the nucleotide specified in the
20   text. When necessary, the concentration of free Mg^{2+} was
adjusted using EDTA buffers as described previously by
Higashijima, 1987, and incorporated herein by reference.

The concentration of free Mg^{2+} in assay medium that
25   contains 5 mM EDTA and no added Mg^{2+} is estimated to be
0.1-0.2 nM according to Brandt et al., 1988 and 1983.
The latter Brandt article also discusses the use of molar
turnover numbers, using GTPγS binding activity to measure
to concentration of total G protein.
30

To measure the MP-catalyzed dissociation of [α-
^{32}P]GDP or .[^{35}S]-GTPγS, labeled nucleotide was first bound
to G protein (approximately 100 mol in 50 μl) by
incubation of the Lubrol-solubilized G protein with a
35   molar excess of nucleotide in assay buffer that contained
10 mM $MgSO_4$ and 0.1% Lubrol. Incubation was for 20 min at
20°C (for G_o) or for 30 min at 30°C (for G_i). In the case

of $[\alpha-^{32}P]GDP$, EDTA was then added to 12 mM. Liganded G
protein was reconstituted by mixing it with 10 μg of
dioleoyl-PC/bovine brain PS/1-palmitoyl,2-oleoyl-PE
(4:3:3) in 50 μl of 50 mM sodium Hepes (pH 8.0), 1 mM
5   dithiothreitol, 0.1% Lubrol 12A9 plus either 12 mM EDTA
and 10 mM $MgSO_4$ (for $[\alpha-^{32}P]GDP$) or 1 mM EDTA and 1.1 mM
$MgSo_4$ (for $[^{35}S]GTP_\gamma S$) and chromatographing the mixture on
a 3-ml column of Ultrogel AcA34 in 50 mM sodium Hepes (pH
8.0), 1 mM EDTA, 1 mM dithiothreitol. The gel filtration
10   during reconstitution removes unbound nucleotide.
Dissociation was initiated at 30°C for G_i or 20°C for G_o
by the addition of G protein-containing vesicles to assay
medium that contained 1 μM unlabeled GDP, GTP, or $GTP_\gamma S$
and free Mg^{2+} at 0.1 mM or other concentrations as shown
15   in the figures. Dissociation was terminated by 5-fold
dilution at 0°C into buffer that contained 0.1% Lubrol
and, in the case of GDP, AMF to stabilize binding.
Bound nucleotide was determined by binding to
nitrocellulose filters as described (*Ferguson*, 1986), and
20   incorporated herein by reference. Dissociation of
nucleotide displayed single component, first order
kinetics. Data were analyzed using a nonlinear least
squares fitting program.

25   The rate of hydrolysis of protein-bound $[\gamma^{32}P]GTP$ was
measured essentially as described previously
(Higashijima, 1987) at 20°C in assay medium that
contained 2 ppm Lubrol 12A9. G_o (10 nM) was incubated in
assay medium for 16 min with 100 nM $[\gamma-^{32}P]GTP$ to allow
30   nucleotide to bind. Hydrolysis was initiated by the
addition of 1.3 mM $MgSO_4$. Excess unlabeled GTP was also
added such that only a single turnover was measured. The
production of $[^{32}P]P_i$ was measured as described by
Higashijima, 1987.

35

The competitive binding of $[^3H]QNB$ and acetylcholine
to reconstituted muscarinic cholinergic receptors was

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measured at 30°C in 100 μl of 20 mM Tris-Cl, pH 8.0, 1mM
EDTA, 2 mM MgSO$_4$, 100 mM NaCl, 1 mM dithiothreitol, 0.1
mg/ml bovine serum albumin. After equilibration for 60
min, vesicles were collected and washed on Whatman GF/F
5   glass fiber filters, as described previously, for the
assay of the ß-adrenergic receptor (Fleming & Ross,
1980), and incorporated herein by reference.

Non-specific binding of [^{3}H]-QNB, which was
10   determined in the presence of 2 μM atropine, was not
altered by MP or by guanine nucleotides.

Intrinsic tryptophan fluorescence was measured as
described by *Higashijima*, 1987.
15

Circular Dichroism

CD spectra of peptides were measured using an Aviv
model 60DS spectrometer at 25°C in 1-mm pathlength cells.
20   Spectra were scanned at 1 nm intervals for 3s, and three
scans were averaged. Peptides were dissolved at 20 μM in
5 mM Tris-Cl, pH 7.5, in the presence or absence of
sonicated 1 mM 1-palmitoyl,2-oleoyl-phosphatidylcholine.
Fractional helical content for each peptide was
25   calculated according to the assumption that, for 100%
helix and 14 peptide bonds, θ_{222} = 36,000 (1 - 2.6/14).

Results

30   *Structure-Activity Relationships for MP Analogs*

Several congeners of MP were synthesized in order to
characterize the structural features that allow it to
catalyze the activation of G proteins. They included
35   both natural peptides that are found in the venoms of
different wasps and nonbiological congeners that were
designed to test the effects of net positive charge, the

spacing and location of charge, and α-helical
conformation on MP's regulatory activity. The structures
of these peptides and their fractional α helical
contents, hydrophobicities, and calculated hydrophobic
5   moments are shown in Table I.

TABLE I

Structure of MP analogs

Analogs in each group displayed virtually identical regulatory activities in the GDP exchange assay shown in Fig. 1. Group VIII analogs were not evaluated in this assay but were slightly more active than group IX analogs in GTPase assays. The hydrophobic moment (μ), a measurement of the asymmetry with which hydrophobicity is distributed around the axis of a helix, was calculated according to Eisenberg et al. (1984) using the hydrophobicity values of Janin (1979). Average hydropathy (HΦ) was calculated using the values of Kyte and Doolittle (1982) without correction for free N-terminal amino groups. Fractional helical contents of some of the peptides were determined by circular dichroism (Fig. 2) in the presence or absence of 1 mM phospholipid using the methods and formula described under "Experimental Procedures." Analogs are numbered arbitrarily except for those noted with letters, which are found in wasp venoms. (Biological MP-A has an Ile at position 13). Hyphens indicate identity with MP.

		Structure	μ	HΦ	% α Helix	
					-PC	+PC
I	MP	INLKALAALAKKIL	0.331	1.16	19	61
II	Mas7	----------AL--	0.273	1.51	26	69
III	Mas8	----------RL--	0.304	1.06	28	79
IV	Mas9	--------A-L--	0.227	1.51	34	67
	10	--------R-L--	0.297	1.06		
	20	---R--------L--	0.292	1.06		
V	Mas19	---A-------L--	0.186	1.51	37	73
VI	MP-A	-KW--ILDAV--V--	0.347	0.61		

	Structure	μ	HΦ	% α Helix −PC	% α Helix +PC
T	-----I--F----L-	0.332	1.09		
X	--W-GI--M----L-	0.322	0.53		
VII Mas14	-------K-----L-	0.462	0.70	16	64
15	-------K------L-	0.372	0.70		
18	---K-------L-	0.376	0.70		
VIII Mas11	--------K--L-	0.223	0.70	20	34
13	--------K---L-	0.278	0.56		
IX Mas6	-----------K-	0.175	0.56	15	31
17	-----K------L-	0.154	0.56		

Their abilities to promote GDP exchange by G_i are
shown in Fig. 1. This assay, which is completed in about
2 min, was used to avoid the need to determine the effect
of each peptide on stability. However, each peptide was
5   also evaluated in a 10-min GTPase assay with similar
results.

Regulatory activity was markedly enhanced by
eliminating the positive charge at position 12 (Mas7, Lys
10   → Ala) or by delocalization of the charge by replacing
lysine with arginine (Mas8). Maximal stimulation by Mas7
was twice as great as stimulation by MP, which typically
stimulates G_i about 15-fold in both GTPase and GDP
exchange assays. The EC_{50} of Mas7 was also 5-fold lower
15   than that of MP. Removal or delocalization of the
positive charges at positions 4 and 11 had similar but
smaller effects (groups IV and V). In general, the
similarity of the effects of the Lys → Arg and Lys → Ala
substitutions were surprising and need to be explored
20   further.

The active MP analogs assumed largely helical
conformations in the presence of phospholipid (Fig. 2 and
Table I), as does MP. Those structural changes that
25   would be predicted to interfere with formation of the
amphipathic α helix or with its binding to the vesicles
decreased regulatory activity. Thus, the peptides in
groups VIII and IX, which have lysine residues on what
should have been the hydrophobic face of the helix, did
30   not form helices in the presence of lipid and were only
slightly active. The group VII peptides, with extra
lysine residues on the hydrophilic face, and the other
natural analogs were only slightly less active than MP.

35   The pattern of potencies and efficacies shown for G_i
in Fig. 2 was similar to that observed for G_o both in
GTPase and GDP exchange assays. Preliminary experiments

suggest that alterations in the hydrophobic face of the
MP structure can alter selectivity between G_i and G_o,
although no obvious structure-function relationship has
emerged from these data.

5

*Structure-Activity Relationships for G Protein
Regulation: Biological Peptides Not Related to MP*

Amphiphilic and cationic peptides are abundant in
10 nature and have diverse biological functions. When an
assortment of peptides was tested for their ability to
activate G_i and G_o, several were found to be quite
effective (Table II).

TABLE II

Effects of biological peptides on the GTPase activity of G_0 and G_1

The GTPase activity of G_1 or G_0 in PE/PC/PS vesicles was assayed in the presence of each peptide for 30 min at 30°C (G_1) or 15 min at 20°C (G_0). Hydrophobic moments (μ) for some peptides and hydrophobicities, $H\Phi$, are calculated as in Table I. -- indicates that 10 μM peptide stimulated exchange by less than 60%. H_1, histidine, not counted toward net charge. Abbreviations used are DALE, [D-Ala2]Leu-enkephalin; FMRF-amide, Phe-Met-Arg-Phe-NH$_2$; LHRH, luteinizing hormone releasing hormone; signal-1, signal peptide of the β subunit of F_1 ATPase (MMITLRKLPLAVAVAAGVMSAQAMA); signal-2, LamB signal peptide (MMITLRKLPLAVAVAAGVMSAQAMA); TRH, thyrotropin releasing hormone; CBP6, Ac-WKKLLKLLKKLLKL-NH$_2$; TRP3, LKWKKLLKLLKKLLKLL-TRH, thyrotropin releasing hormone; CBP6, Ac-WKKLLKLLKKLLKL-NH$_2$; TRP3, LKWKKLLKLLKKLLKLL-TRH, thyrotropin releasing hormone; CBP6, Ac-WKKLLKLLKKLLKL-NH$_2$; TRP3, LKWKKLLKLLKKLLKLL-KLG. *, Orn was assigned the hydrophobicity of Lys.

Peptide	GTPase				μ	Net Charge	$H\Phi$
	G_o		G_1				
	10 μM	100μM	10μM	100μM			
	min^{-1}						
None	0.06		0.03				
Mastoparan	0.59	0.95	0.19	0.47	0.331	+4	1.1
CBP6	0.76	0.38	0.51	0.06	0.712	+6	0.1
TRP3	0.63	0.54	0.44	0.09	0.523	+7	0.1
Melittin	0.63	0.56	0.37	0.40	0.239	+6	0.4
Gramicidin S	0.17	0.47	0.13	0.31	Cyclic	+2	1.0*

Peptide	GTPase				μ	Net Charge	H%
	G_o		G_i				
	10 μM	100μM	10μM	100μM			
Dynorphin	0.13	0.33	-	0.10	0.202	+4	-1.2
Signal-2	-	0.36	-	0.06	0.065	+2	1.3
Angiotensin II	-	0.31	-	0.23	0.096	0,H	-0.3
Substance P	-	0.25	-	0.09	0.093	+3	-0.7
Signal-1	-	0.22	-	0.08	0.245	+4	0.1
α - Mating factor	-	0.19	-	0.05	0.138	+2,H	-0.8
Calcitonin	-	0.09	-	0.01	0.139	+1,H	0.0
Glucagon	-	0.08	-	0.06	0.102	0,H	-0.9
FMRF-amide	-	0.07	-	0.04	0.511	+2	0.7
TRH	-	0.06	-	0.03	0.134	0,H	-2.7
DALE	-	0.06	-	0.02	0.026	0	1.3
Melanostatin	-	0.05	-	0.03	0.258	+1	0.6
Bradykinin	-	0.05	-	0.03	0.165	+2	-1.0
LHRH	-	0.04	-	0.03	0.349	+1,H	-1.2

5

10

15

CBP6 and TRP3, two synthetic peptides that were designed to form cationic, amphiphilic α helices were quite active. Melittin, an amphiphilic peptide composed of a hydrophobic region and a cationic hydrophilic region, activated both G proteins with potency and efficacy roughly equivalent to that of mastoparan. Gramicidin S and dynorphin were also somewhat stimulatory at 10 μM. AT higher concentrations, peptides such as angiotensin II, yeast α mating factor, and two signal peptides all stimulated GTPase activity more than 3-fold. Substance P was able to stimulate G_i and G_o more than 4-fold, which is consistent with the proposal that the "non-receptor-mediated" effects of substance P on mast cell secretion may reflect its MP-like stimulation of a G protein.

As a cautionary note, it should be pointed out that almost all of the peptides that were tested caused at least minor stimulation at high concentrations. Activities of the weaker peptides did not correlate closely with either net charge, charge per length or hydrophobic moment. Because even hydrophobic amines (see below) and ammonium sulfate can promote nucleotide exchange on G proteins, arguments for specific structure-function relationships and analogies to the structures of specific receptors must be based on careful studies of concentration dependence and on correlation between regulation of cell function and *in vitro* stimulation of G proteins.

Inhibition of MP Effects of G_i by BAC

The existence of competitive antagonists has traditionally been used to support the existence of specific binding sites for regulatory ligands. BAC is a hydrophobic quaternary amine and antibacterial agent that is known to block histamine secretion from mast cells

stimulated by compound 48/80 BAC also antagonized the stimulatory effect of Mas7 and MP on the GTPase activity of G_i (Figs. 3 and 4). Inhibition was reversible and appeared to be at least partly competitive. However, BAC destablizes G proteins, and the effect of high concentrations of BAC on steady state GTPase activity could not be conveniently studied because the assays became nonlinear.

The concentration-dependent antagonist activity of BAC was analysed using the rapid equilibrium GDP/GDP exchange assay (Fig. 4). At intermediate concentrations of Mas7, BAC inhibited GDP exchange on G_i by as much as 60%, to a final rate that was still about 5-fold greater than the rate measured in the absence of regulator or in the presence of BAC alone. Inhibition was overcome at higher concentrations of Mas7. There was no detectable inhibition of the basal exchange rate, and high concentrations of BAC alone stimulated basal GDP exchange slightly (Fig. 4).

Stimulation of Nucleotide Exchange on G_o by Hydrophobic Amines

In contrast to its MP-antagonist activity on G_i, BAC and several other quaternary amines actually stimulated the nucleotide exchange and GTPase activities of G_o (Fig. 5 and Table III).

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TABLE III

Effects of BAC and other alkylamines on G_o and G_l

The GTPase activity of G_l or G_o in PE/PC/PS vesicles was assayed in the presence of each amine under the conditions described in the legend to Table II. No effect of amines was observed at 1 μg/ml. BAC (benzalkonium chloride), benzyldimethylakylammonium chloride with alkyl chain length shown. The C_{12} BAC (Sigms) is heterogeneous. Me, methyl; Bz, benzyl; Bu, butyl.

Amine	Turnover number			
	G_o		G_l	
	10 μg/ml	100 μg/ml	10 μg/ml	100 μg/ml
	min⁻¹			
None	0.07		0.04	
BAC (C_{12})	0.33	0.8	0.08	0.04
BAC (C_{14})	0.9	0.2	0.04	<0.01
BAC (C_{16})	1.4	0.08	0.04	<0.01
BAC (C_{18})	1.1	0.3	0.03	<0.01
BzBu$_3$NCl	0.07	0.07	0.04	0.04
C_{12}Me$_3$NCl	0.1	0.5	0.04	0.04
C_{18}Me$_3$NCl	0.61	<0.01	0.04	<0.01
C_{12}NH$_2$Cl	0.18	0.61	0.06	0.31

Stimulation was dependent on the concentration of phospholipid, as was true for MP. In the experiment shown in Fig. 5, 20-fold maximal stimulation was observed at about 30 μg/ml (~0.1 mM) BAC. Hydrophobic amines markedly destablized G proteins, however, and regulation was difficult to quantitate at high concentrations. Above 30 μg/m BAC, the apparent rate of $[\alpha-^{32}P]$GDP release increased sharply because of denaturation, and the GTPase rate declined, even when measured over only 1 min. Stimulation by BAC was also slight and not well reproducible unless the G protein was reconstituted into phospholipid vesicles.

The requirement for reconstitution may reflect both actual stabilization of G_o by the lipid and the ability of the bilayer to buffer the local concentration of detergent.

Several amines stimulated the GTPase activity of G_o (Table III). Among the BACs, increasing alkyl chain length increased potency but did not have an obvious effect on maximal stimulation. Potency may reflect the tendency of the BAC to partition into the bilayer at low concentrations. The concentration-dependent stimulation of the GDP exchange rate by different BACs, which would provide more informative data on maximum effects, has not yet been elucidated. Other long chain alkylamines, primary through quaternary, stimulated G_o, but short chain amines were ineffective. Of the compounds tested, only dodecylamine stimulated G_i significantly.

Apparent Positive Cooperativity of G Protein Regulation by Cationic Amphiphiles

Under most experimental conditions, MP and other amphiphilic G protein regulators exerted their effects over a narrow range of concentrations (Figs. 1 and 4B).

The concentration dependence on BACs for the stimulation
of G_o was also sharp (Table III and Fig. 5). These steep
responses are described by Hill coefficient of 2-4 for MP
and its analogs. Examples for Mas7 are shown in Fig. 4B.
Such responses suggest that 2-4 molecules of amphiphilic
peptide may be required for the productive regulation of
G protein. These apparently cooperative effects of
stimulatory and inhibitory amphiphiles may indicate
either that multiple molecules must bind to distinct
sites on a G protein or that the formation of a dimer,
trimer, or tetramer is required for activity, whether
stimulatory or inhibitory. Spontaneous formation of an
oligomer of strongly cationic species seems unlikely
because the concentrations of MP used here are well below
that at which aggregates form. Practically, changes in
nucleotide exchange rates over narrow ranges of
concentration have made detailed kinetic analyses
difficult. It is likely that the mechanism that
underlies the high Hill coefficient will have to be
determined by independent physical probes.

The inhibitory effect of BAC on G, also yielded a
steep concentration curve, as determined by monitoring
the concentration of BAC needed to decrease the potency
with which Mas7 stimulated nucleotide exchange (Fig. 4B).
The EC_{50} for Mas7 increased sharply in the range of 1-3
μg/ml BAC. However, both inhibition by BAC of the
stimulation of G_i by MP (Fig. 4) and maximal stimulation
of G_o by BAC (Fig. 5) occurred near the critical micelle
concentration of 20 μg/ml, and their interpretation is
therefore unclear.

*Effect of MP on the G Protein-induced Increase in the
Affinity of Muscarinic Receptors for Agonists*

G proteins increase the affinity of receptors for
agonist, but not antagonist, ligands. This increase

reflects the formation of a G protein-receptor-agonist
complex. It is reversed by addition of excess guanine
nucleotide or, frequently, by solubilization of the
membrane in which the receptor and G protein reside. If
5   MP binds to G proteins at or near the receptor binding
site, it might be expected to interfere with the
formation of the complex. Fig. 6 shows the effect of MP
on the binding of acetylcholine to purified muscarinic
cholinergic receptor that was co-reconstituted into
10   phospholipid vesicles with G_o. Binding was measured by
competition with the antagonist [^{3}H]QNB. Both MP and a
highly active MP analog, Mas7, decreased the receptor's
affinity for acetylcholine to nearly the same extent as
did 10 μm $GTP_{\gamma}S$. An inactive analog of MP, Mas17, had no
15   effect on acetylcholine binding. If receptor was
reconstituted into vesicles without G protein, only low
affinity binding of acetylcholine was observed, and
neither MP nor guanine nucleotides had any effect on
acetylcholine binding. MP did not alter the affinity of
20   the receptor for the antagonist [^{3}H]QNB or the shape of
the [^{3}H]QNB binding isotherm when assayed using receptor-
G_o vesicles.

DISCUSSION

25

MP and related G protein regulators are potentially
useful as cellular probes of G protein function and as
structural models of the G protein-activating domains of
the much larger receptors. To fulfill this promise,
30   however, the mechanism of their action must be understood
and the structural determinants of their potency and
selectively among G proteins must be identified.

Mechanism -By enzymological criteria, MP facilitates
35   nucleotide exchange by a mechanism similar to that
utilized by G protein-coupled receptors. MP promoted the
dissociation of either GDP or GTP_{γ} from either G_i or G_o in

the virtual absence of Mg^{2+} and did not alter the rate of hydrolysis of bound GTP. The effect of MP on the dissociation of $GTP\gamma$ was much smaller than its effect on the dissociation GTP. This phenomenon is consistent with the knowledge that GDP competes poorly with $GTP\gamma S$ for mascarinic receptor-stimulated binding to G_o.

The Mg^{2+}-independent effects of MP on GDP release are adequate to account for the stimulation by MP of steady state GTPase activity at submicromolar Mg^{2+}. Similar dependencies of GTPase activity on low concentrations of Mg^{2+} have been observed for the stimulation of G_s by the β-adrenergic receptor, of $G_{1,2}$ by the D_2 dopamine receptor, and of G_1 by the muscarinic cholinergic receptor.

The Mg^{2+} independence of MP-stimulated nucleotide exchange suggests that the only requirement for Mg^{2+} in the overall GTPase cycle is in the conversion of the G protein-GTP complex to the activated form. This step immediately precedes hydrolysis of the bound GTP, and it is assumed that hydrolysis also requires bound Mg^{2+}. The interacting effects of Mg^{2+} and MP on GTPase activity suggest that there are two sites at which Mg^{2+} binds G proteins. Binding at the high affinity site allows activation and hydrolysis, and binding at the low affinity site promotes nucleotide exchange in the absence of MP (or receptor).

The inhibitory effect of high concentrations of Mg^{2+} on MP-stimulated steady state GTPase activity and MP-stimulated dissociated of GDP from free α subunit remains unexplained.

Structures of Regulatory Compounds-The data of Fig. 1 and Tables I and II delineate structural features that are required for catalysis of nucleotide exchange on G proteins. Regulatory activity appears to require a

minimum length, positive charge, and a combination of net
hydrophobicity and hydrophobic moment that is sufficient
to allow binding to the bilayer in a way that orients the
charge toward the G protein. In the case of Mp and its

5   analogs, this cluster of charge is apparently formed from
one face of an amphiphilic helix whose folding is induced
when MP binds to a micelle or vesicle (Fig. 2). Other
secondary structures can also form such cationic clusters
at the surface of bilayers, however.

10

Within the MP analogs, increasing either
hydrophobicity or hydrophobic moment enhanced potency and
maximal regulatory activity, although the dominance of
one parameter over the ocher was not clear. The most

15   active analogs were those in which Lys was replaced by
either Arg or Ala, with substitution at position 12 being
most effective. It was surprising that these two
replacements for lysine were approximately equal in
activity. the increased activity of the Arg-containing

20   analogs may reflect the greater delocalization of charge
in the guanidino group of arginine relative to the
primary amino group of lysine. Delocalization of charge
increases functional hydrophobicity. Thus, while the
Lys→Ala substitutions increased hydrophobicity more than

25   Lys→Arg, the Lys→Arg substitutions would delocalize one
of the positive charges and also maintain a high
hydrophobic moment. Similarly, CBP6, TRP3, and melittin
are far less hydrophobic than the MP analogs according to
amino acid composition, but their strong amphiphilicity

30   causes them to be membrane-bound and to present a high
cationic charge density at the bilayer surface.

It is likely that G proteins are stimulated
specifically by the α-helical conformation of MP which

35   forms readily at the surface of micelles or bilayers but
not in aqueous solution. Thus, the group VIII and IX
analogs, with charge oriented toward what would be the

wrong side of the helix, were far less active than MP.
They did not form α helices in the presence of
phospholipids (Fig. 2, Table I). The group VII analogs,
with one extra charge oriented toward the putative
hydrophilic face, did form α helices and were similar to
MP in regulatory activity. The dependence of P potency
on the concentration of phospholipid also implies that
the surface of the bilayer is actually the relevant
solvent for these peptides.

Mp and other amphiphilic G protein regulators are
attractive as cellular probes for the signaling
activities of individual G proteins. Although a
concentration of positive charge at the surface of the
bilayer is apparently necessary for the catalysis of
nucleotide exchange by G proteins, the different
responses of G_i and G_o to the compounds tested here argue
for structural specificity and the possibility of
designing compounds that will be highly selective among
the homologous G proteins. The observation that BAC
stimulates G_o but antagonizes the effect of MP on G_i is a
strong argument for specific and selective binding of
cationic regulators to G proteins. Because BAC inhibits
the stimulation of histamine secretion from mast cells,
this finding also suggests that G_i is the most plausible
target of MP in these cells. Another example of
selectivity is that G_s and transducin are far less
sensitive to MP than are G_o and G_i, while a Trp,Arg
copolymer strongly stimulates G_s but stimulates G_i and G_o
only slightly. These data all suggest that amphiphilic,
cationic peptides bind to a negatively charged site (or
sites) on G proteins, but that the detailed structure of
the site is selective among cationic ligands.

The regulation of G proteins by MP and by receptors
appear to be similar in many ways. If MP actually is a
structural analog of the regulatory domain of G protein-

coupled receptors, then MP should compete with receptors
for binding to a common site on G proteins. Such a model
predicts data of the sort shown in Fig. 6 because MP
should compete for G_o with the receptor, a known regulator
5   of G_o and thus block the ability of G_o to enhance the
affinity with which the receptor binds agonists. The
data of Fig. 6, while provocative, do not prove that MP
and the receptor are actually competing for G_o. Minimal
proof would require demonstrating that increasing
10   concentrations of G_o can appropriately overcome the
effects of increasing concentrations of MP. These
experiments are limited by the need for high
concentrations of G protein to regulate the binding of
the mascarinic receptor and by the relatively low potency
15   of the currently available MP analogs. Competition
between receptors and MP for binding to G protein will
more likely be demonstrated through direct binding assays
or the use of MP-based antagonists.

20   The analogy between the MPs and the intracellular
loops of the G protein-coupled receptors suggests that a
cationic, amphipathic structure formed from one or more
of these loops comprises the activating site on a
receptor's cytoplasmic surface. This idea is consistent
25   with the results of genetic and chemical manipulations of
these domains in the receptors. We do not know whether
this functionally defined "site" corresponds to a single
structural region on a receptor or whether several
cationic regions must be appropriately presented for G
30   protein regulation to occur. The latter idea would
account for the high ill coefficients that we observed
for the MPs and is consistent with the ability of
independent mutations into short regions of the M1-
muscarnic cholinergic receptor to alter selectivity
35   between G proteins. Regardless, the ability to design MP
analogs with varying activities and selectivities and the
relative ease with which their three-dimensional

structure can be determined argue for their continued application to the question.

EXAMPLE 2

This example relates to the regulation of G_o, G_i and G_s by modified mastoparan-based peptides and synthetic peptides corresponding to intracellular loops of G protein-receptors. In particular, the present example provides an investigation and analysis of various peptides of the invention, along with their apparent affinity and efficacy.

G-protein-coupled receptors share a structural motif which is characterized by 7 hydrophobic domains, thought to represent membrane-spanning helices, connected by more hydrophilic extracellular and intracellular loops. Genetic and biochemical analyses of several of these receptors suggests that the intracellular loop domains mediate the coupling of the receptors with G proteins. Deletion mutagenesis and hybrid receptor analysis have implicated regions at the N- and C-terminal ends of the third intracellular loop as the major, but not the sole, determinants of G protein coupling.

Secondary structure predictions suggested to the inventors that the regions at the N- and C-terminal ends of this loop may be α-helical in nature, forming amphipathic cytoplasmic extensions of transmembrane helices 5 and 6. However, there is no consensus amino acid sequence in this region from which coupling to a specific G protein can be predicted. The lack of primary sequence homology in this region among receptors which couple to the same G protein has led to the hypothesis that it is the amphiphilic and cationic nature of these α-helical regions that is the main determinant in the interaction of receptors with G proteins. This is in

keeping with the structure and activity of the mastoparans, which form amphiphilic helices, parallel to the plane of the membrane, when binding to a phospholipid bilayer.

The following studies were designed to demonstrate and further delineate the structure-activity relationships for the regulation of G proteins by mastoparans, and to compare their activity with synthetic peptides corresponding to intracellular loops of various receptors. Furthermore, the effect of specific modifications on the activity of the receptor-based peptides was investigated.

The data set forth in the following tables represent data generated by the inventors employing the steady state GTP hydrolysis, expressed as molar turnover numbers, are described herein under the "Assay" section of the Experimental Procedures in Example 1. The data has been classified according to the criteria outlined below and the effects of each peptide and modified variant on G_o, G_i, and G_s have been tabulated. Table III below sets forth data generated for various mastoparan analogues designed by the inventors, in comparison with the various naturally-occurring mastoparans (e.g., MP, MP-A and MP-T). Table IV sets forth similar data generated for various receptor-based peptides designed by the inventors based on a consideration of various G-protein linked receptors.

Apparent affinity (af.) and apparent efficacy (ef.) were estimated by EC_{50} and maximum turnover number of GTPase activity.

For affinity:
   Super (S): less than 3 μM
   Excellent (E): 3-10 μM

Good (G):   10-30 μM

Fair (F):   30-100 μM

Poor (P):   more than 100 μM

5   For efficacy:

Super (S):   more than 30-fold

Excellent (E):   30-10 fold

Good (G):   5-10 fold

Fair (F):   2-5 fold

10   Poor (P):   less than 2 fold

For MAS peptides, GDP-dissociation as well as GTP-
hydrolysis were observed, and basically similar results

15   were obtained.

TABLE IV

			Go$_o$		G$_1$		G$_s$	
			af.	ef.	af.	ef.	af.	ef.
	MP	INLKALAALAKKIL-NH$_2$	E	E	G	E	E	F
5	MP-A	-KW--ILDAV--V-	E	E	E	G	E	F
	MP-T	-----I--F---L-	E	E	E	E	G	F
	MAS03	-----------L-	E	E	G	E	G	F
	MAS04	-----------LA	E	E	G	G	E	F
	MAS05	-----------A-	G	G	G	F	G	F
10	MAS06	-----------K-	F	F	F	F	F	F
	MAS07	----------AL-	E	S	E	S	E	F
	MAS08	----------RL-	E	E	E	E	E	F
	MAS09	---------A-L-	E	E	E	E	F	F
	MAS10	---------R-L-	E	E	G	E	E	F
15	MAS11	--------K--L-	G	G	E	F	E	F
	MAS12	--------A---L-	G	E	F	G	E	F
	MAS13	--------K---L-	F	G	F	G	E	F
	MAS14	-------K----L-	G	E	E	G	E	F
	MAS15	------K-----L-	G	E	G	G	E	F
20	MAS16	-----A------L-	G	E	F	G	E	F
	MAS17	-----K------L-	F	F	P	P	E	F
	MAS18	----K-------L-	G	E	G	G	E	F
	MAS19	---A--------L-	E	E	E	E	E	F
	MAS20	---R--------L-	E	E	E	E	E	F
25	MAS21	--A---------L-	G	E	F	E	G	F
	MAS22	--F---------L-	G	E	G	E	E	F
	MAS23	----F-------L-	G	E	G	E	E	F
	MAS24	-----------L-	E	E	G	E	G	F

TABLE IV (cont.)

		G_o		G_1		G_s	
		af.	ef.	af.	ef.	af.	ef.
ER1	INLKALAALAKALL-NH$_2$	E	S	E	S	E	F
ER2	----------R---	S	E	E	S	E	F
ER3	----------Q---	G	E	G	S	E	F
ER4	---A----------	E	E	G	S	E	F
ER5	----------A---	E	E	G	S	E	F
ER6	---R----------	S	E	E	S	E	F
ER7	---R------R---	S	S	E	S	E	F
ER8	---Q----------	E	S	E	S	E	F
ER9	---Q------Q---	F	G	P	F	G	F
ER10	------K-------	S	S	E	S	E	F
ER11	---Q--K-------	E	E	E	S	E	F
ER12	-Q------------	E	E	E	S	E	F
ER13	--Y--------KI-	E	E	G	E	E	F
ER14	--Y-----C--KI-	E	E	G	E	E	F
ER15	--Y---C----KI-	F	G	G	G	G	F
ER16	C-Y----------	E	E	E	S	G	F
C12-ER16		S	E	S	S	E	F
C16-ER16		S	E	S	S	E	F
ER17	--Y----------C	E	E	E	E	G	F
C12-ER17		S	E	S	S	E	F
C16-ER17		S	E	S	E	E	F
ER20	-----------N--	E	E	G	E	G	F
ER21	-L------------	E	E	E	S	E	E
ER24	---R--R---R---	S	E	E	S	E	F
ER25	--------K----	G	E	G	E	G	F
ER27	--WR-WR-W-R-W-	S	P	E	G	E	P
ER28	---R-----RRR--	S	G	G	F	E	P
ER29	--------K-K-- (=MAS 11)	G	G	G	G	E	F
ER30	CL------------	E	G	E	E	E	E
C12-ER30		S	G	S	E	E	F
ER26	AcLTAVLLTLTLLLYRINL KALAALAKALL-NH$_2$	S	F	S	G	E	P

TABLE V
Sequences and Explanation of Receptor-Based Peptides

ER18	CVYREAKEQIRKIDRVL-NH$_2$	turkey ß-I3N
ER19	CVYREAKEQIRKIL-NH$_2$	turkey ß-I3N
ER22	IVYREAKEQIRKIDRVL-NH$_2$	turkey ß-I3N
ER23	IVYREAKEQIRKIL-NH$_2$	turkey ß-I3N
ER32	CIYRETENRARELAALQGSET-NH$_2$	M1-I3N
ER33	CIYRETENRARELAALQGSETIL-NH$_2$	M1-I3N
ER34	CVYIVAKRTTKNLEAGVMKEI-NH$_2$	Ha-α1-I3N
ER35	CVYIVAKRTTKNLEIL-NH$_2$	Ha-α1-I3N
ER36	CVFQVAKRQLQKIDKVL-NH$_2$	Ha-ß2-I3N
ER37	Ac-ER-36	Ha-ß2-I3N
ER38	CPLSYRAKRTPRRAALM-NH$_2$	M1-I2
ER39	CPFRYQSLNTRARAKVI-NH$_2$	turkey ß-I2
ER40	REHKALKTLGIIC-NH$_2$	turkey ß-I3C
ER41	CRSPDFRKAPKRLLC-NH$_2$	turkey ß-I4N
ER42	Ac-ER-41	turkey B-I4N
ER43	CVYREAKEQIRKIDR-NH$_2$	turkey ß-I3N w/o IL
ER44	CISRASKSRIKKDKKEPVAIL-NH$_2$	Ms-I3N
ER45	CISRASKSRIKKDKKEPVAIL-NH$_2$	M2-I3N
ER46	CVYVVAKRESRGLKSGLKTDIL-NH$_2$	COW A1-I3N
ER47	CVYVVAKRESRGLKIL-NH$_2$	COW A1-I3N

TABLE VI

		G_o		G_1		G_s	
		af.	ef.	af.	ef.	af.	ef.
	ER18	P	F	G	F	P	F
	C12-ER18	G	F	G	G	E	E
5	ER19	P	F	G	F	P	P
	C12-ER19	G	F	G	G	E	E
	ER22	F	F	F	F	F	F
	ER23	F	F	F	F	F	F
	ER32	P	P	P	P	P	P
10	C12-ER32	P	P	P	P	P	P
	ER33	P	P	P	P	P	P
	C12-ER33	P	P	P	P	P	P
	ER34	F	F	G	F	F	F
	C12-ER34	S	G	E	G	F	G
15	ER35	G	F	F	F	P	P
	C12-ER35	G	F	G	F	G	F
	ER36	G	F	G	F	F	E
	C12-ER36	S	G	S	G	E	G
	ER38	E	F	G	F	F	F
20	C12-ER38	G	F	E	G	F	P
	ER39	F	F	P	P	F	F
	C12-ER39	E	G	E	G	F	F
	ER40	P	P	P	P	F	F
	C12-ER40	E	F	E	G	E	F
25	ER43	P	P	P	P	P	P
	C12-ER43	G	F	G	F	G	F
	ER44	S	F	P	P	F	F
	C12-ER44	S	F	E	F	F	F
	ER45	S	F	P	P	E	F
30	C12-ER45	G	F	E	F	F	F
	ER46	F	F	E	F	P	P
	C12-ER46	F	F	G	G	F	F
	ER47	F	F	P	F	P	P
	C12-ER47	P	P	G	P	F	F

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*   *   *

The present invention has been described in terms of particular embodiments found or proposed by the present inventors to comprise preferred modes for the practice of the invention. It will be appreciated by those of skill in the art that, in light of the present disclosure, numerous modifications and changes can be made in the particular embodiments exemplified without departing from the intended scope of the invention. All such modifications are intended to be included within the scope of the appended claims.

REFERENCES

The following references, to the extent that they supplement, explain or provide a basis for, techniques disclosed or referred to herein are hereby incorporated by reference

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SEQUENCE LISTING

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    (iii) TITLE OF INVENTION: METHODS AND COMPOSITIONS FOR
                  MODULATING G PROTEIN ACTION

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        (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: WordPerfect 5.1

    (vii) CURRENT APPLICATION DATA:
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        (B) FILING DATE: Unknown
        (C) CLASSIFICATION: Unknown

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        (C) CLASSIFICATION: 514

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(2) INFORMATION FOR SEQ ID NO:1:

        (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 14 amino acids
             (B) TYPE: amino acid
             (C) STRANDEDNESS: unknown
             (D) TOPOLOGY: unknown

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

        Ile Asn Leu Lys Ala Leu Ala Ala Leu Ala Lys Lys Leu Leu
        1   5   10

(2) INFORMATION FOR SEQ ID NO:2:

        (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 14 amino acids
             (B) TYPE: amino acid
             (C) STRANDEDNESS: unknown
             (D) TOPOLOGY: unknown

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

        Ile Asn Leu Lys Ala Leu Ala Ala Leu Ala Lys Lys Leu Ala
        1   5   10

(2) INFORMATION FOR SEQ ID NO:3:

        (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 14 amino acids
             (B) TYPE: amino acid
             (C) STRANDEDNESS: unknown
             (D) TOPOLOGY: unknown

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

        Ile Asn Leu Lys Ala Leu Ala Ala Leu Ala Lys Lys Ala Leu
        1   5   10

(2) INFORMATION FOR SEQ ID NO:4:

        (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 14 amino acids
             (B) TYPE: amino acid
             (C) STRANDEDNESS: unknown
             (D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Ile Asn Leu Lys Ala Leu Ala Ala Leu Ala Lys Lys Lys Leu
1   5   10

(2) INFORMATION FOR SEQ ID NO:5:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 14 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: unknown
        (D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Ile Asn Leu Lys Ala Leu Ala Ala Leu Ala Lys Ala Leu Leu
1   5   10

(2) INFORMATION FOR SEQ ID NO:6:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 14 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: unknown
        (D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Ile Asn Leu Lys Ala Leu Ala Ala Leu Ala Lys Arg Leu Leu
1   5   10

(2) INFORMATION FOR SEQ ID NO:7:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 14 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: unknown
        (D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Ile Asn Leu Lys Ala Leu Ala Ala Leu Ala Ala Lys Leu Leu
1   5   10

(2) INFORMATION FOR SEQ ID NO:8:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 14 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: unknown
        (D) TOPOLOGY: unknown

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
Ile Asn Leu Lys Ala Leu Ala Ala Leu Ala Arg Lys Leu Leu
1               5                   10
```

(2) INFORMATION FOR SEQ ID NO:9:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 14 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: unknown
        (D) TOPOLOGY: unknown

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```
Ile Asn Leu Lys Ala Leu Ala Ala Leu Lys Lys Lys Leu Leu
1               5                   10
```

(2) INFORMATION FOR SEQ ID NO:10:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 14 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: unknown
        (D) TOPOLOGY: unknown

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
Ile Asn Leu Lys Ala Leu Ala Ala Ala Ala Lys Lys Leu Leu
1               5                   10
```

(2) INFORMATION FOR SEQ ID NO:11:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 14 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: unknown
        (D) TOPOLOGY: unknown

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Ile Asn Leu Lys Ala Leu Ala Ala Lys Ala Lys Lys Leu Leu
1   5   10

(2) INFORMATION FOR SEQ ID NO:12:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 14 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: unknown
        (D) TOPOLOGY: unknown

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Ile Asn Leu Lys Ala Leu Ala Lys Leu Ala Lys Lys Leu Leu
1   5   10

(2) INFORMATION FOR SEQ ID NO:13:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 14 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: unknown
        (D) TOPOLOGY: unknown

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Ile Asn Leu Lys Ala Leu Lys Ala Leu Ala Lys Lys Leu Leu
1   5   10

(2) INFORMATION FOR SEQ ID NO:14:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 14 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: unknown
        (D) TOPOLOGY: unknown

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

```
Ile Asn Leu Lys Ala Ala Ala Ala Leu Ala Lys Lys Leu Leu
1               5                   10
```

(2) INFORMATION FOR SEQ ID NO:15:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 14 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: unknown
        (D) TOPOLOGY: unknown

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

```
Ile Asn Leu Lys Ala Lys Ala Ala Leu Ala Lys Lys Leu Leu
1               5                   10
```

(2) INFORMATION FOR SEQ ID NO:16:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 14 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: unknown
        (D) TOPOLOGY: unknown

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

```
Ile Asn Leu Lys Lys Leu Ala Ala Leu Ala Lys Lys Leu Leu
1               5                   10
```

(2) INFORMATION FOR SEQ ID NO:17:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 14 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: unknown
        (D) TOPOLOGY: unknown

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Ile Asn Leu Ala Ala Leu Ala Ala Leu Ala Lys Lys Leu Leu
1   5   10

(2) INFORMATION FOR SEQ ID NO:18:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 14 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: unknown
        (D) TOPOLOGY: unknown

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Ile Asn Leu Arg Ala Leu Ala Ala Leu Ala Lys Lys Leu Leu
1   5   10

(2) INFORMATION FOR SEQ ID NO:19:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 14 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: unknown
        (D) TOPOLOGY: unknown

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Ile Asn Ala Lys Ala Leu Ala Ala Leu Ala Lys Lys Leu Leu
1   5   10

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(2) INFORMATION FOR SEQ ID NO:20:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 14 amino acids
       (B) TYPE: amino acid
       (C) STRANDEDNESS: unknown
       (D) TOPOLOGY: unknown

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

```
Ile Asn Phe Lys Ala Leu Ala Ala Leu Ala Lys Lys Leu Leu
1               5                   10
```

(2) INFORMATION FOR SEQ ID NO:21:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 14 amino acids
       (B) TYPE: amino acid
       (C) STRANDEDNESS: unknown
       (D) TOPOLOGY: unknown

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

```
Ile Asn Leu Lys Ala Phe Ala Ala Leu Ala Lys Lys Leu Leu
1               5                   10
```

(2) INFORMATION FOR SEQ ID NO:22:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 14 amino acids
       (B) TYPE: amino acid
       (C) STRANDEDNESS: unknown
       (D) TOPOLOGY: unknown

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

```
Ile Asn Leu Lys Ala Leu Ala Ala Leu Ala Lys Ala Leu Leu
1               5                   10
```

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(2) INFORMATION FOR SEQ ID NO:23:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 14 amino acids
            (B) TYPE: amino acid
            (C) STRANDEDNESS: unknown
            (D) TOPOLOGY: unknown

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

        Ile Asn Leu Lys Ala Leu Ala Ala Leu Ala Arg Ala Leu Leu
        1   5   10

(2) INFORMATION FOR SEQ ID NO:24:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 14 amino acids
            (B) TYPE: amino acid
            (C) STRANDEDNESS: unknown
            (D) TOPOLOGY: unknown

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

        Ile Asn Leu Lys Ala Leu Ala Ala Leu Ala Gln Ala Leu Leu
        1   5   10

(2) INFORMATION FOR SEQ ID NO:25:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 14 amino acids
            (B) TYPE: amino acid
            (C) STRANDEDNESS: unknown
            (D) TOPOLOGY: unknown

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

        Ile Asn Leu Ala Ala Leu Ala Ala Leu Ala Lys Ala Leu Leu
        1   5   10

(2) INFORMATION FOR SEQ ID NO:26:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 14 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: unknown
        (D) TOPOLOGY: unknown

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

```
Ile Asn Leu Lys Ala Leu Ala Ala Leu Ala Ala Ala Leu Leu
1               5                   10
```

(2) INFORMATION FOR SEQ ID NO:27:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 14 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: unknown
        (D) TOPOLOGY: unknown

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

```
Ile Asn Leu Arg Ala Leu Ala Ala Leu Ala Lys Ala Leu Leu
1               5                   10
```

(2) INFORMATION FOR SEQ ID NO:28:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 14 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: unknown
        (D) TOPOLOGY: unknown

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

```
Ile Asn Leu Arg Ala Leu Ala Ala Leu Ala Arg Ala Leu Leu
1               5                   10
```

(2) INFORMATION FOR SEQ ID NO:29:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 14 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: unknown
        (D) TOPOLOGY: unknown

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

```
Ile Asn Leu Gln Ala Leu Ala Ala Leu Ala Lys Ala Leu Leu
1               5               10
```

(2) INFORMATION FOR SEQ ID NO:30:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 14 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: unknown
        (D) TOPOLOGY: unknown

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

```
Ile Asn Leu Gln Ala Leu Ala Ala Leu Ala Gln Ala Leu Leu
1               5               10
```

(2) INFORMATION FOR SEQ ID NO:31:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 14 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: unknown
        (D) TOPOLOGY: unknown

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

```
Ile Asn Leu Lys Ala Leu Lys Ala Leu Ala Lys Ala Leu Leu
1               5               10
```

(2) INFORMATION FOR SEQ ID NO:32:

        (i) SEQUENCE CHARACTERISTICS:
              (A) LENGTH: 14 amino acids
              (B) TYPE: amino acid
              (C) STRANDEDNESS: unknown
              (D) TOPOLOGY: unknown

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

        Ile Asn Leu Gln Ala Leu Lys Ala Leu Ala Lys Ala Leu Leu
        1   5   10

(2) INFORMATION FOR SEQ ID NO:33:

        (i) SEQUENCE CHARACTERISTICS:
              (A) LENGTH: 14 amino acids
              (B) TYPE: amino acid
              (C) STRANDEDNESS: unknown
              (D) TOPOLOGY: unknown

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

        Ile Gln Leu Lys Ala Leu Ala Ala Leu Ala Lys Ala Leu Leu
        1   5   10

(2) INFORMATION FOR SEQ ID NO:34:

        (i) SEQUENCE CHARACTERISTICS:
              (A) LENGTH: 14 amino acids
              (B) TYPE: amino acid
              (C) STRANDEDNESS: unknown
              (D) TOPOLOGY: unknown

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

        Ile Asn Tyr Lys Ala Leu Ala Ala Leu Ala Lys Lys Ile Leu
        1   5   10

(2) INFORMATION FOR SEQ ID NO:35:

    (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 14 amino acids
      (B) TYPE: amino acid
      (C) STRANDEDNESS: unknown
      (D) TOPOLOGY: unknown

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

Ile Asn Tyr Lys Ala Leu Ala Ala Cys Ala Lys Lys Ile Leu
1   5   10

(2) INFORMATION FOR SEQ ID NO:36:

    (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 14 amino acids
      (B) TYPE: amino acid
      (C) STRANDEDNESS: unknown
      (D) TOPOLOGY: unknown

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

Ile Asn Tyr Lys Ala Leu Cys Ala Leu Ala Lys Lys Ile Leu
1   5   10

(2) INFORMATION FOR SEQ ID NO:37:

    (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 14 amino acids
      (B) TYPE: amino acid
      (C) STRANDEDNESS: unknown
      (D) TOPOLOGY: unknown

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

Cys Asn Tyr Lys Ala Leu Ala Ala Leu Ala Lys Ala Leu Leu
1   5   10

(2) INFORMATION FOR SEQ ID NO:38:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 14 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: unknown
        (D) TOPOLOGY: unknown

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

    Ile Asn Tyr Lys Ala Leu Ala Ala Leu Ala Lys Ala Leu Cys
    1   5   10

(2) INFORMATION FOR SEQ ID NO:39:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 14 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: unknown
        (D) TOPOLOGY: unknown

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

    Ile Asn Leu Lys Ala Leu Ala Ala Leu Ala Lys Asn Leu Leu
    1   5   10

(2) INFORMATION FOR SEQ ID NO:40:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 14 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: unknown
        (D) TOPOLOGY: unknown

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

    Ile Leu Leu Lys Ala Leu Ala Ala Leu Ala Lys Ala Leu Leu
    1   5   10

(2) INFORMATION FOR SEQ ID NO:41:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 14 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: unknown
        (D) TOPOLOGY: unknown

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

Ile Asn Leu Arg Ala Leu Arg Ala Leu Ala Arg Ala Leu Leu
1   5   10

(2) INFORMATION FOR SEQ ID NO:42:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 14 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: unknown
        (D) TOPOLOGY: unknown

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

. Ile Asn Leu Lys Ala Leu Ala Ala Leu Lys Lys Ala Leu Leu
1   5   10

(2) INFORMATION FOR SEQ ID NO:43:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 14 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: unknown
        (D) TOPOLOGY: unknown

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

Ile Asn Trp Arg Ala Trp Arg Ala Trp Ala Arg Ala Trp Leu
1   5   10

(2) INFORMATION FOR SEQ ID NO:44:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 14 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: unknown
        (D) TOPOLOGY: unknown

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

    Ile Asn Leu Arg Ala Leu Ala Ala Leu Arg Arg Arg Leu Leu
    1   5   10

(2) INFORMATION FOR SEQ ID NO:45:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 14 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: unknown
        (D) TOPOLOGY: unknown

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

    Ile Asn Leu Lys Ala Leu Ala Ala Leu Lys Lys Lys Leu Leu
    1   5   10

(2) INFORMATION FOR SEQ ID NO:46:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 14 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: unknown
        (D) TOPOLOGY: unknown

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

    Cys Leu Leu Lys Ala Leu Ala Ala Leu Ala Lys Ala Leu Leu
    1   5   10

(2) INFORMATION FOR SEQ ID NO:47:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 26 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: unknown
        (D) TOPOLOGY: unknown

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

Leu Thr Ala Val Leu Leu Thr Leu Leu Leu Tyr Arg Ile Asn Leu Lys
1   5   10   15

   Ala Leu Ala Ala Leu Ala Lys Ala Leu Leu
        20   25

CLAIMS:

1.   A peptide other than MP, MP-A or MP-T, the peptide
being from 14 to 26 amino acids in length and including
within its structure a G protein modulator region
comprising:

    a)   a mastoparan analog region having the following
          formula:

$$AA_1-AA_2-AA_3-AA_4-AA_5-AA_6-AA_7-AA_8-AA_9-AA_{10}-AA_{11}-AA_{12}$$

    wherein:

AA_1 = L, W, A, F, Y or W;
AA_2 = K, A, R or Q;
AA_3 = A or K;
AA_4 = L, W, I, A, K or F;
AA_5 = A, L, K, C or R;
AA_6 = A, D or K;
AA_7 = L, C. W, A, F or K;
AA_8 = A, V, K or R;
AA_9 = K, R, Q or A;
AA_{10} = K, A, R or N;
AA_{11} = L, I. W, V, A or K; and
AA_{12} = L, A or C; or

    b)   a receptor analog region comprising
          CVYREAKEQIRKIDRVL; CVYREAKEQIRKIL;
          IVYREAKEQIRKIDRVL; IVYREAKEQIRKIL;
          CIYRETENRARELAALQGSET; CIYRETENRARELAALQGSETIL;
          CVYIVAKRTTKNLEAGVMKEIL; CVYIVAKRTTKNLEIL;
          CPLSYRAKRTPRRAALM; CPFRYQSLMTRARAKVI;
          REHKALKTLGIIC; CRSPDFRKAFKRLLC;
          CVYREAKEQIRKIDR; CISRASKSRIKKDKKEPVAIL;
          CISRASKSRIKKDKKIL; CVYVVAKRESRGLKSGLKTDIL; or
          CVYVVAKRESRGLKIL.

2.   The peptide of claim 1, wherein the peptide comprises a G protein modulator mastoparan analog region having the following structure:

$$AA_A-AA_B-AA_1-AA_2-AA_3-AA_4-AA_5-AA_6-AA_7-AA_8-AA_9-AA_{10}-AA_{11}-AA_{12}$$

wherein:

AA_A = I or C;

AA_B = N, K, Q or L;

AA_1 = L, W, A, F, Y or W;

AA_2 = K, A, R or Q;

AA_3 = A or K;

AA_4 = L, W, I, A, K or F;

AA_5 = A, L, K, C or R;

AA_6 = A, D or K;

AA_7 = L, C. W, A, F or K;

AA_8 = A, V, K or R;

AA_9 = K, R, Q or A;

AA_{10} = K, A, R or N;

AA_{11} = L, I. W, V, A or K; and

AA_{12} = L, A or C.

3.   The peptide of claim 1, wherein the analog region comprises LKALAALAKKLL.

4.   The peptide of claim 1, wherein the analog region comprises LKALAALAKKLA.

5.   The peptide of claim 1, wherein the analog region comprises LKALAALAKKAL.

6.   The peptide of claim 1, wherein the analog region comprises LKALAALAKKKL.

 5   7.   The peptide of claim 1, wherein the analog region comprises LKALAALAKALL.

 8.   The peptide of claim 1, wherein the analog region
10   comprises LKALAALAKRLL.

 9.   The peptide of claim 1, wherein the analog region comprises LKALAALAAKLL.

15

10.   The peptide of claim 1, wherein the analog region comprises LKALAALARKLL.

20

11.   The peptide of claim 1, wherein the analog region comprises LKALAALKKKLL.

25   12.   The peptide of claim 1, wherein the analog region comprises LKALAAAAKKLL.

13.   The peptide of claim 1, wherein the analog region
30   comprises LKALAAKAKKLL.

14.   The peptide of claim 1, wherein the analog region comprises LKALAKLAKKLL.

35

15. The peptide of claim 1, wherein the analog region comprises LKALKALAKKLL.

5   16. The peptide of claim 1, wherein the analog region comprises LKAAAALAKKLL.

17. The peptide of claim 1, wherein the analog region
10   comprises LKAKAALAKKLL.

18. The peptide of claim 1, wherein the analog region comprises LKKLAALAKKLL.

15

19. The peptide of claim 1, wherein the analog region comprises LAALAALAKKLL.

20

20. The peptide of claim 1, wherein the analog region comprises LRALAALAKKLL.

25   21. The peptide of claim 1, wherein the analog region comprises AKALAALAKKLL.

22. The peptide of claim 1, wherein the analog region
30   comprises FKALAALAKKLL.

23. The peptide of claim 1, wherein the analog region comprises LKAFAALAKKLL.

35

24.   The peptide of claim 1, wherein the analog region comprises LKALAALAKALL.

5   25.   The peptide of claim 1, wherein the analog region comprises LKALAALARALL.

26.   The peptide of claim 1, wherein the analog region
10   comprises LKALAALAQALL.

27.   The peptide of claim 1, wherein the analog region comprises LAALAALAKALL.

15

28.   The peptide of claim 1, wherein the analog region comprises LKALAALAAALL.

20

29.   The peptide of claim 1, wherein the analog region comprises LRALAALAKALL.

25   30.   The peptide of claim 1, wherein the analog region comprises LRALAALARALL.

31.   The peptide of claim 1, wherein the analog region
30   comprises LQALAALAKALL.

32.   The peptide of claim 1, wherein the analog region comprises LQALAALAQALL.

35

33. The peptide of claim 1, wherein the analog region comprises LKALKALAKALL.

34. The peptide of claim 1, wherein the analog region comprises LQALKALAKALL.

35. The peptide of claim 1, wherein the analog region comprises QLKALAALAKALL.

36. The peptide of claim 1, wherein the analog region comprises YKALAALAKKIL.

37. The peptide of claim 1, wherein the analog region comprises YKALAACAKKIL.

38. The peptide of claim 1, wherein the analog region comprises YKALCALAKKIL.

39. The peptide of claim 1, wherein the analog region comprise CNYKALAALAKALL.

40. The peptide of claim 1, wherein the analog region comprises YKALAALAKALC.

41. The peptide of claim 1, wherein the analog region comprises LKALAALAKNLL.

42.   The peptide of claim 1, wherein the analog region comprises ILLKALAALAKALL.

5   43.   The peptide of claim 1, wherein the analog region comprises LRALRALARALL.

44.   The peptide of claim 1, wherein the analog region
10   comprises LKALAALKKALL.

45.   The peptide of claim 1, wherein the analog region comprises WRAWRAWARAWL.

15

46.   The peptide of claim 1, wherein the analog region comprises LRALAALRRRLL.

20

47.   The peptide of claim 1, wherein the analog region comprises LKALAALKKKLL.

25   48.   The peptide of claim 1, wherein the analog region comprises CLLKALAALAKALL.

49.   The peptide of claim 1, wherein the analog region
30   comprises LTAVLLTLLLYRINLKALAALAKALL.

50.   The peptide of claim 1, wherein the peptide comprises a G protein modulator that includes a receptor
35   peptide region having the following structure:

CVYREAKEQIRKIDRVL; CVYREAKEQIRKIL;

IVYREAKEQIRKIDRVL; IVYREAKEQIRKIL;

CIYRETENRARELAALQGSET; CIYRETENRARELAALQGSETIL;

CVYIVAKRTTKNLEAGVMKEIL; CVYIVAKRTTKNLEIL;

CPLSYRAKRTPRRAALM; CPFRYQSLMTRARAKVI; REHKALKTLGIIC;

CRSPDFRKAFKRLLC; CVYREAKEQIRKIDR;

CISRASKSRIKKDKKEPVAIL; CISRASKSRIKKDKKIL;

CVYVVAKRESRGLKSGLKTDIL; or CVYVVAKRESRGLKIL.

51. The peptide of claim 50, wherein the receptor peptide region comprises CVYREAKEQIRKIDRVL.

52. The peptide of claim 50, wherein the receptor peptide region comprises CVYREAKEQIRKIL.

53. The peptide of claim 50, wherein the receptor peptide region comprises IVYREAKEQIRKIDRVL.

54. The peptide of claim 50, wherein the receptor peptide region comprises IVYREAKEQIRKIL.

55. The peptide of claim 50, wherein the receptor peptide region comprises CIYRETENRARELAALQGSET.

56. The peptide of claim 50, wherein the receptor peptide region comprises CIYRETENRARELAALQGSETIL.

57. The peptide of claim 50, wherein the receptor peptide region comprises CVYIVAKRTTKNLEAGVMKEIL.

58.   The peptide of claim 50, wherein the receptor peptide region comprises CVYIVAKRTTKNLEIL.

59.   The peptide of claim 50, wherein the receptor peptide region comprises CPLSYRAKRTPRRAALM.

60.   The peptide of claim 50, wherein the receptor peptide region comprises CPFRYQSLMTRARAKVI.

61.   The peptide of claim 50, wherein the receptor peptide region comprises REHKALKTLGIIC.

62.   The peptide of claim 50, wherein the receptor peptide region comprises CRSPDFRKAFKRLLC.

63.   The peptide of claim 50, wherein the receptor peptide region comprises CVYREAKEQIRKIDR.

64.   The peptide of claim 50, wherein the receptor peptide region comprises CISRASKSRIKKDKKEPVAIL.

65.   The peptide of claim 50, wherein the receptor peptide region comprises CISRASKSRIKKDKKIL.

66.   The peptide of claim 50, wherein the receptor peptide region comprises CVYVVAKRESRGLKSGLKTDIL.

67. The peptide of claim 50, wherein the receptor peptide region comprises CVYVVAKRESRGLKIL.

5   68. The peptide of claim 1, wherein the first two amino terminal amino acids comprise hydrophobic amino acids.

69. The peptide of claim 68, wherein the first two amino
10   terminal amino acids comprise I, L or V.

70. The peptide of claim 1, amidated at its carboxy terminus.

15

71. The peptide of claim 1, acylated at its amino terminus.

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72. The peptide of claim 71, wherein the acylated amino terminus is acylated through an amino terminal cysteine residue.

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73. The peptide of claim 71, wherein the amino terminus is acylated with a C-10 to C-22 alkyl group.

30   74. The peptide of claim 73, wherein the amino terminus is alkylated with a C-12 to C-16 alkyl group.

75. The peptide of claim 1, comprising a hydrophobic C-
35   terminus.

76. The peptide of claim 75, wherein hydrophobic C-terminus comprises a hydrophobic dipeptide region.

77. The peptide of claim 76, wherein the hydrophobic dipeptide region comprises I, V or L residues.

78. The peptide of claim 1, wherein at least one amino acid of said analog comprises a D-isomer.

79. The peptide of claim 1, further defined as capable of activating a G-protein.

80. The peptide of claim 1, wherein AA_2, AA_9 or AA_{10} comprises alanine, glutamine or arginine.

81. The peptide of claim 80, wherein A_{10} comprises alanine.

82. The peptide of claim 80, wherein more than one of AA_2, AA_9 and AA_{10} comprises alanine, glutamine or arginine.

83. A pharmacologic composition comprising a therapeutically effective amount of a peptide comprising a mastoparan analog in accordance with claim 1, dispersed in a pharmacologically acceptable carrier or diluent.

84. The composition of claim 83, wherein the analog is dispersed in a carrier adapted for topical administration.

85. A method for modulating G protein action in a cell
comprising subjecting the cell to an amount of a peptide
in accordance with claim 1 that is effective to modulate
5   the action of one or more G proteins.
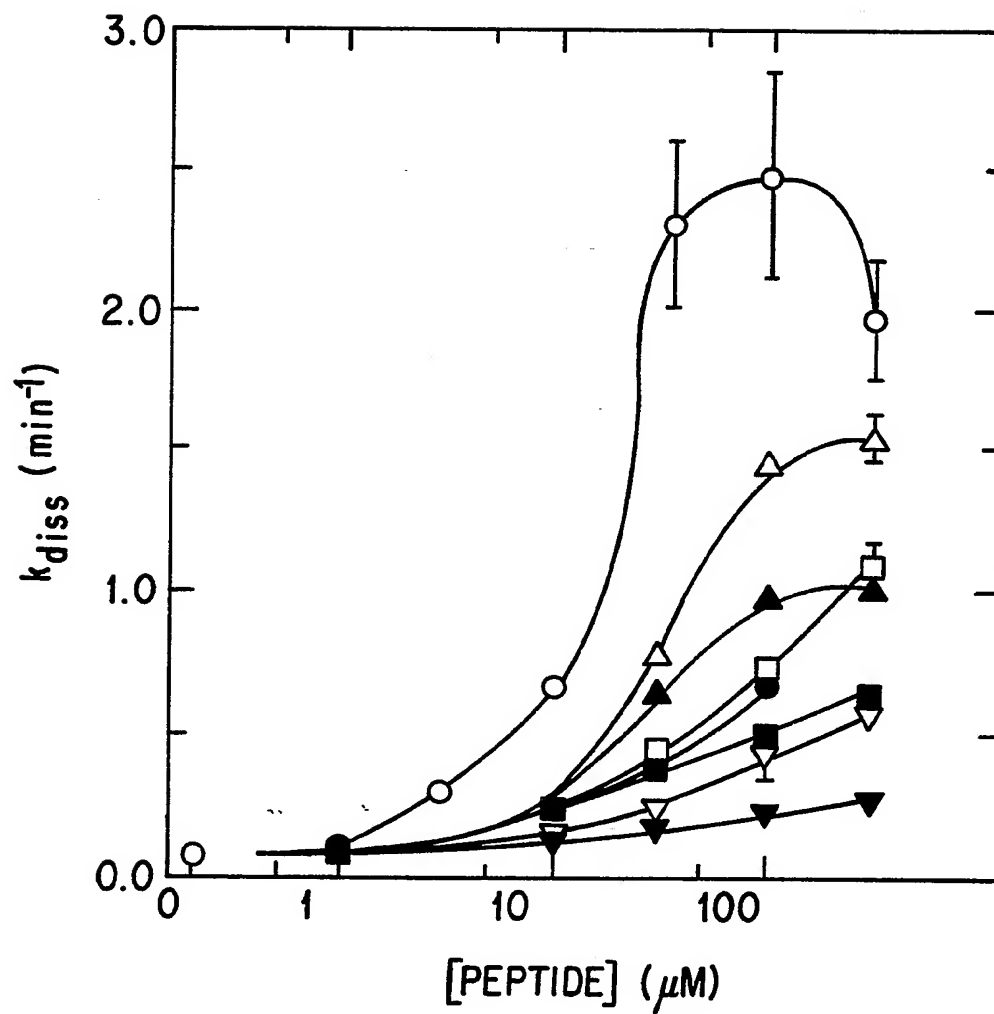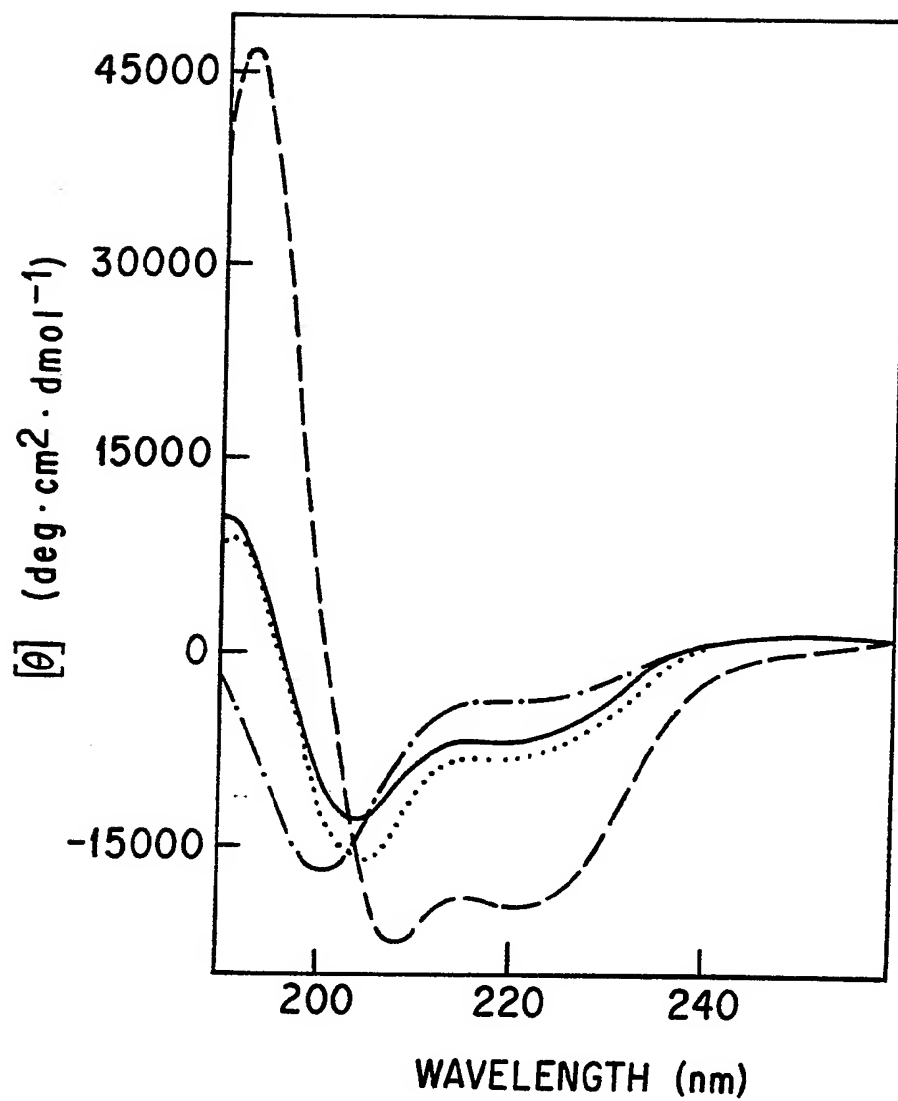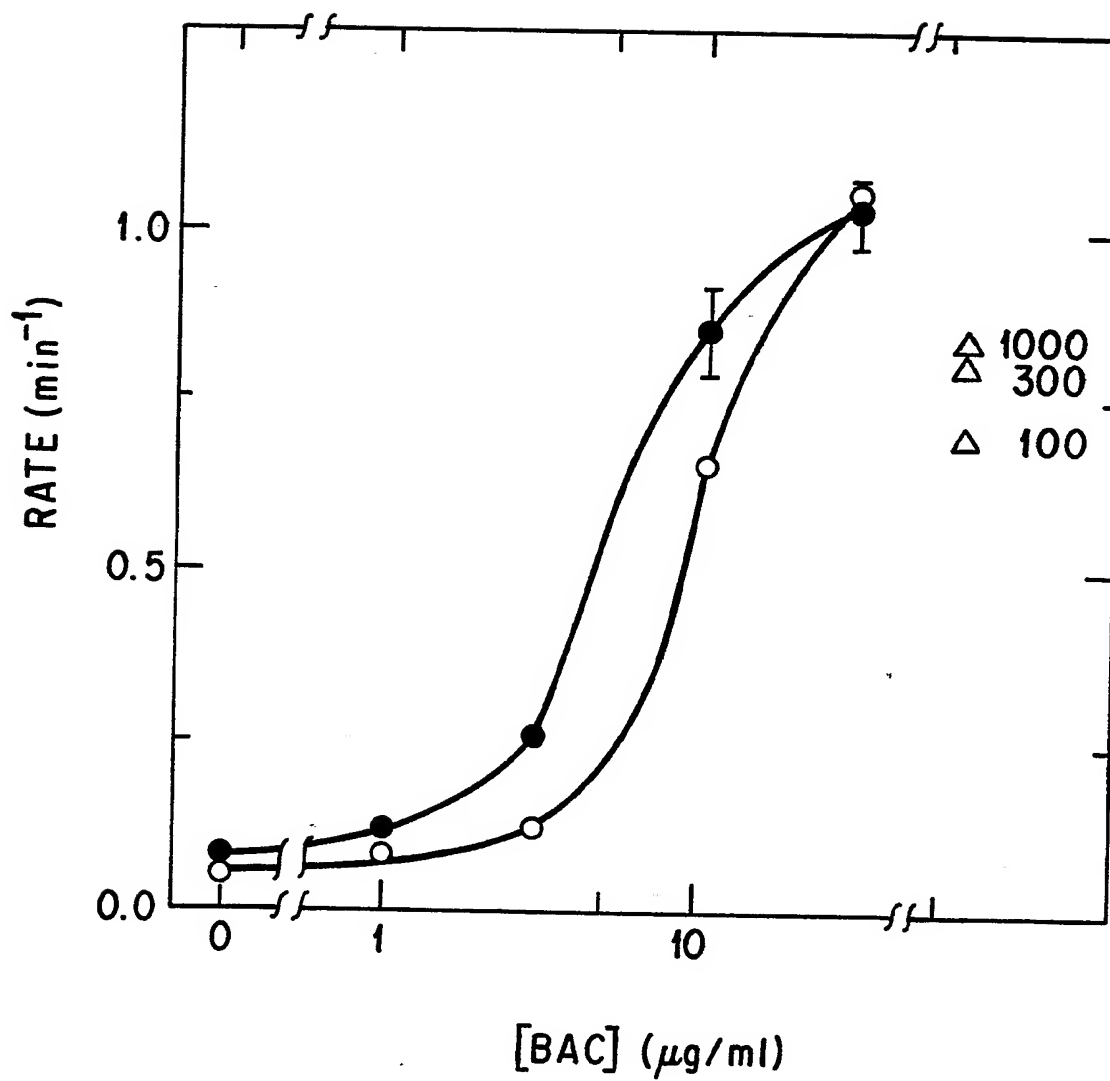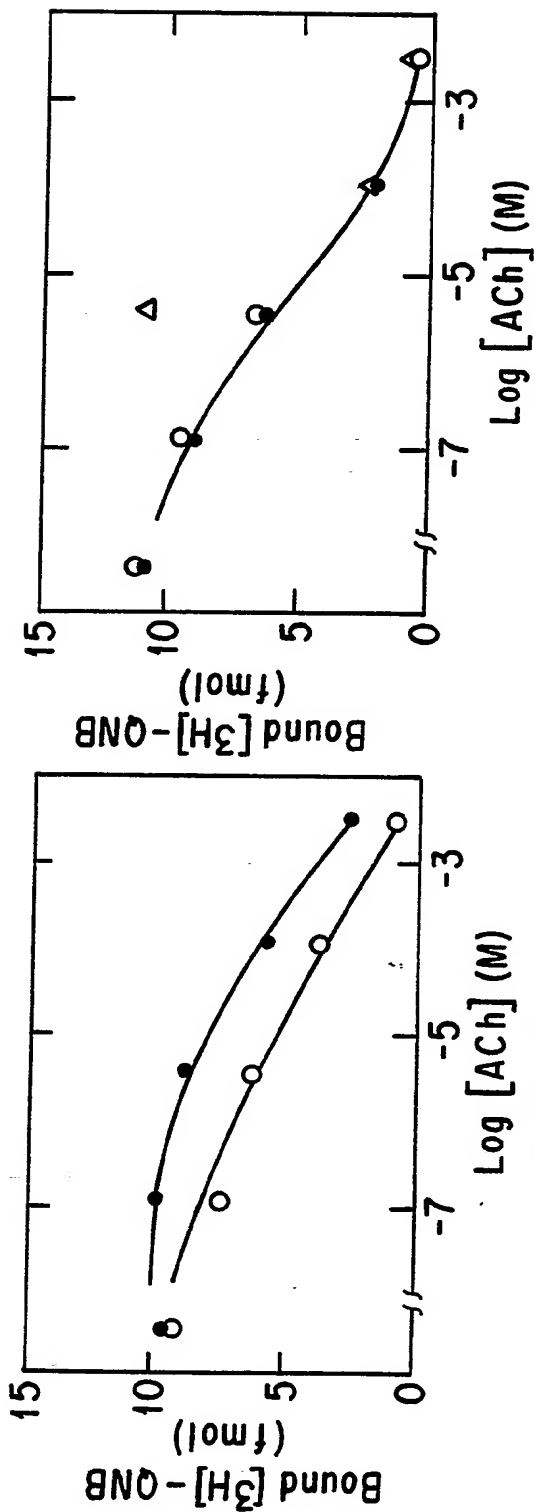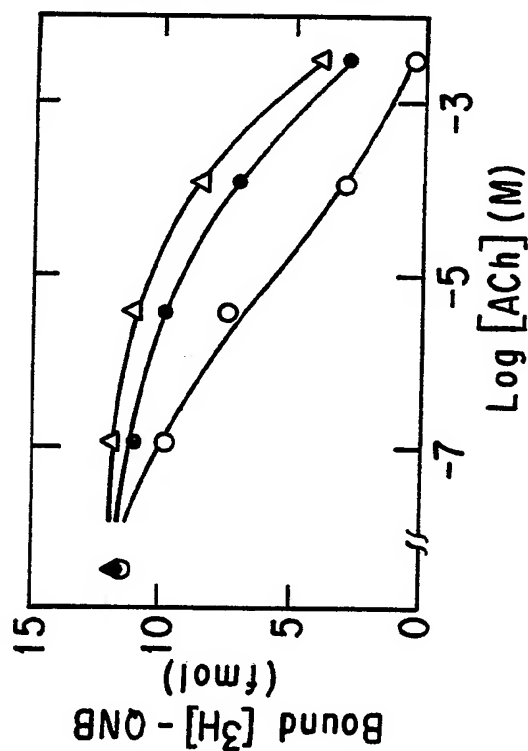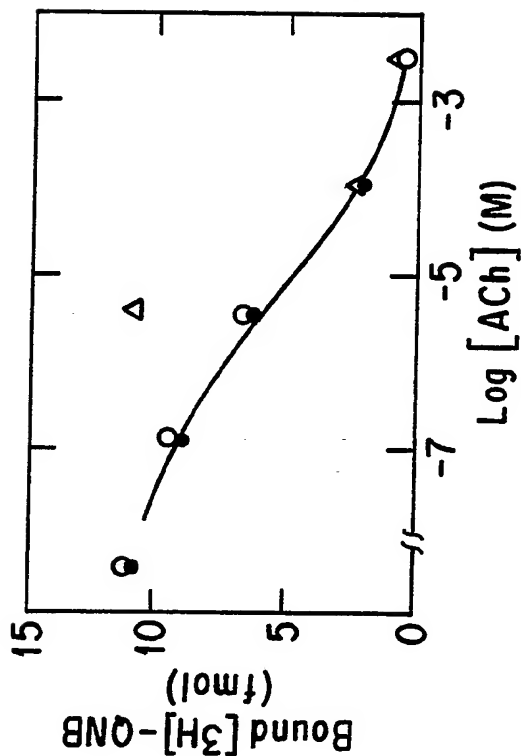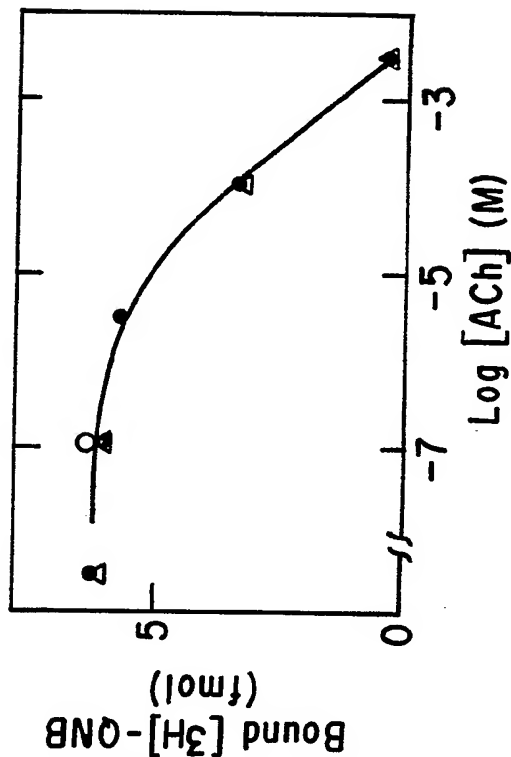
FIG. 1

FIG. 2

FIG. 3
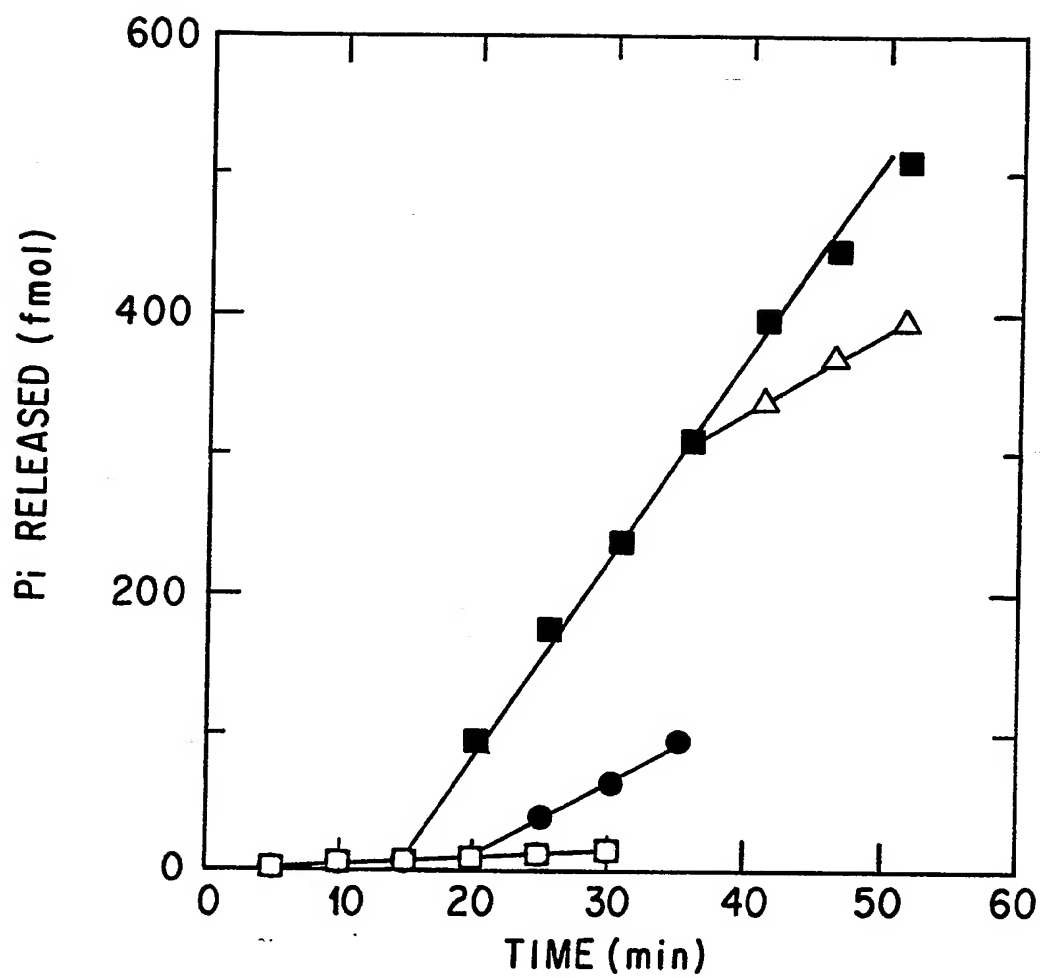
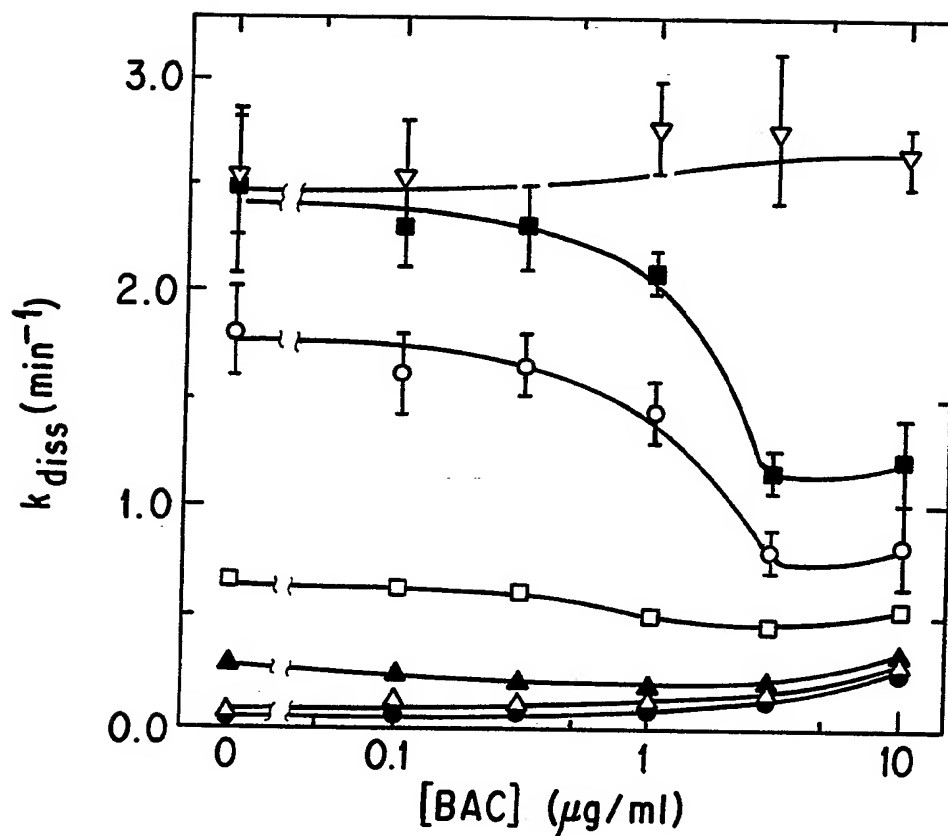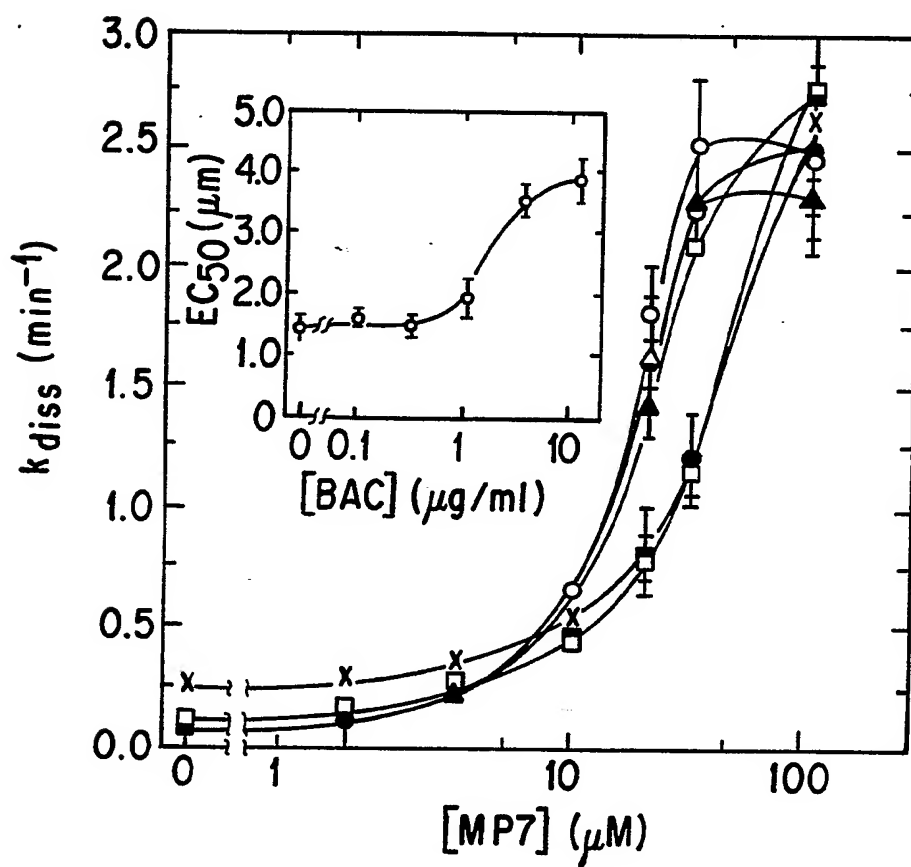FIG. 4A

FIG. 4B

FIG. 5

FIG. 6B

FIG. 6D

FIG. 6A

FIG. 6C

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC(5) :A61K 37/02; C07K 7/08, 7/10
US CL :530/326,327; 514/13, 14
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/326,327; 514/13, 14

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Cas Online File reg, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Journal of Biological Chemistry, vol 258, No 22, issued 25 November 1983, Argiolas et al, "Facilitation of Phospholippase A2 Activity by Mastpparans, a New Class of Mast Cell Degranulating Peptides from Wasp Venom", pages 13697-13702, see pages 13697-702.	1-85
A	Chem. Pharm. Bull, vol 28, no 4. issued 1980, Yajima et al, "Studies on Peptides LXXXVI Application of the Trifluoracetic Acid-Thioanisole Deprotecting Procedure for the Synthesis of A Wasp Venom, Mastoparan", pages 1214-18. see pages 1214-18.	1-85

☐ Further documents are listed in the continuation of Box C.   ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance		
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"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)		
		"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents. such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure. use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
09 NOVEMBER 1992	

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